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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 July 2001 (26.07.2001)

PCT

(10) International Publication Number
WO 01/52791 A2

(51) International Patent Classification[7]: A61K

(21) International Application Number: PCT/US01/01781

(22) International Filing Date: 18 January 2001 (18.01.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/488,393 20 January 2000 (20.01.2000) US

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(81) Designated States *(national)*: AU, CA, JP.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: COMPLEXES OF PEPTIDE-BINDING FRAGMENTS OF HEAT SHOCK PROTEINS AND THEIR USE AS IMMUNOTHERAPEUTIC AGENTS

(57) Abstract: The present invention relates to pharmaceutical compositions comprising peptide-binding fragments of heat shock proteins (HSPs) and noncovalent complexes of peptide-binding fragments of HSPs in noncovalent association with antigenic molecules. The invention further relates to methods for the use of such pharmaceutical compositions as immunotherapeutic agents for the treatment and prevention of infectious diseases and cancer.

A.

```
  1   *   10    *   20    *   30    *   40    *   50
  1 GKIIGIDLGTTNSCVAIMDGTTPRVLENAEGDRTTPSIIAYTQDGETLVG
 51 QPAKRQAVTNPQNTLFAIKRLIGRRFQDEEVQRDVSIMPFKIIAADNGDA
101 WVEVKGQKMAPPQISAEVLKKMKKTAEDYLGEPVTEAVITVPAYFNDAQR
151 QATKDAGRIAGLEVKRIINEPTAAALAYGLDKGTGNRTIAVYDLGGGTFD
201 ISIIEIDEVDGEKTFEVLATNGDTHLGGEDFDSRLINYLVEEFKKDQGID
251 LRNDPLAMQRLKEAAEKAKIELSSAQQTDVNLPYITADATGPKHMNIKVT
301 RAKLESLVEDLVNRSIEPLKVALQDAGLSVSDIDDVILVGGQTRMPMVQK
351 KVAEFFGKEPRKDVNPDEAVAIGAAVQGGVLTGDVKDVLLLDVTPLSLGI
401 ETMGGVMTTLIAKNTTIPTKHSQVFSTAEDNQSAVTIHVLQGERKRAADN
451 KSLGQFNLDGINPAPRGMPQIEVTFDIDADGILHVSAKDKNSGKEQKITI
501 KASSGLNEDEIQKMVRDAEANAEADRKFEELVQTRNQGDHLLHSTRKQVE
551 EAGDKLPADDKTAIESALTALETALKGEDKAAIEAKMQELAQVSQKLMEI
601 AQQQHAQQQTAGADASANNAKDDDVVDAEFEEVKDKK
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B.

```
    1   *   10    *   20    *   30    *   40    *   50
  1 MKLSLVAAMLLLLSAARAEEEDKKEDVGTVVGIDLGTTYSCVGVFKNGRV
 51 EIIANDQGNRITPSYVAFTPEGERLIGDAAKNQLTSNPENTVFDAKRLIG
101 RTWNDPSVQQDIKFLPFKVVEKKTKPYIQVDIGGGQTKTFAPEEISAMVL
151 TKMKETAEAYLGKKVTHAVVTVPAYFNDAQRQATKDAGTIAGLNVMRIIN
201 EPTAAAIAYGLDKREGEKNILVFDLGGGTFDVSLLTIDNGVFEVVATNGD
251 THLGGEDFDQRVMEHFIKLYKKKTGKDVRKDNRAVQKLRREVEKAKALSS
301 QHQARIEIESFYEGEDFSETLTRAKFEELNMDLFRSTMKPVQKVLEDSDL
351 KKSDIDEIVLVGGSTRIPKIQQLVKEFFNGKEPSRGINPDEAVAYGAAVQ
401 AGVLSGDQDTGDLVLLHVCPLTLGIETVGGVMTKLIPSNTVVPTKNSQIF
451 STASDNQPTVTIKVYEGERPLTKDNHLLGTFDLTGIPPAPRGVPQIEVTF
501 EIDVNGILRVTAEDKGTGNKNKITITNDQNRLTPEEIERMVNDAEKFAEE
551 DKKLKERIDTRNELESYAYSLKNQIGDKEKLGGKLSSEDKETMEKAVEEK
601 IEWLESHQDADIEDFKAKKKELEEIVQPIISKLYGSAGPPPTGEEDTAEK
651 DEL
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WO 01/52791 A2

COMPLEXES OF PEPTIDE-BINDING FRAGMENTS OF HEAT SHOCK PROTEINS AND THEIR USE AS IMMUNOTHERAPEUTIC AGENTS

This invention was made with government support under grant numbers CA44786 and CA64394 awarded by the National Institutes of Health. The government has certain rights in the invention.

1. INTRODUCTION

The present invention relates to pharmaceutical compositions comprising peptide-binding fragments of heat shock proteins (HSPs) and peptide-binding fragments of HSPs in noncovalent association with antigenic molecules. The invention further relates to methods for the use of such pharmaceutical compositions as immunotherapeutic agents for the treatment and prevention of infectious diseases and cancer.

2. BACKGROUND OF THE INVENTION

2.1. Heat Shock Proteins

Heat shock proteins (HSPs), also referred to interchangeably as stress proteins, were first identified as proteins synthesized by a cell in response to heat shock. To date, five major classes of HSPs have been identified, based on the molecular weight of the family members. These classes are called sHSPs (small heat shock proteins), Hsp60, Hsp70, Hsp90, and Hsp100, where the numbers reflect the approximate molecular weight of the HSPs in kilodaltons.

Many HSPs have been found to be induced in response to stressful stimuli other than heat, including nutrient deprivation, metabolic disruption, oxygen radicals, and infection with intracellular pathogens (see Welch, May 1993, Scientific American 56-64; Young, 1990, Annu. Rev. Immunol. 8:401-420; Craig, 1993, Science 260:1902-1903; Gething *et al.*, 1992, Nature 355:33-45; and Lindquist *et al.*, 1988, Annu. Rev. Genetics 22:631-677).

HSPs are involved not only in cellular protection against adverse conditions, but are also involved in essential biochemical and immunological processes in unstressed cells. For example, HSPs are involved in various kinds of chaperoning functions. Members of the Hsp70 family, located in the cell cytoplasm, nucleus, mitochondria, or endoplasmic reticulum are involved in the presentation of antigens to the cells of the immune system, and are also involved in the transfer, folding and assembly of proteins in normal cells

(Lindquist *et al.*, 1988, Ann. Rev. Genetics 22:631-677). A number of proteins thought to be involved in chaperoning are residents of the endoplasmic reticulum (ER) lumen, for example, protein disulfide isomerase (PDI; Gething *et al.*, 1992, Nature 355:33-45), Grp94 or ERp99 (Sorger and Pelham, 1987, J. Mol. Biol., 194:991-94) which is related to Hsp90, and Grp78 or BiP, which is related to Hsp70 (Munro *et al.*, 1986, Cell 46:291-300; Haas & Webl, 1983, Nature 306:387-389). These proteins are known to bind a variety of mutant, unfolded, incompletely glycosylated proteins (Machamer *et al.*, 1990, J. Biol. Chem. 65:6879-6883; Gething *et al.*, 1986, Cell 46:939-950).

Heat shock proteins bind polypeptides for their various cellular activities. The binding and release of polypeptides is facilitated by adenosine triphosphate (ATP), at least in the Hsp70 and Hsp90, and possibly other, families of proteins. ATP hydrolysis accompanies polypeptide binding, followed by the subsequent release of the polypeptide in Hsp70 (Flynn *et al.*, 1989, Science, 245:385-390) and Hsp90 (Obermann *et al.*, J. Cell. Biol., 1998, 143:901-10). Structural analysis of DnaK, the Hsp70 family protein of *E. coli*, suggests that a conformational change in the protein structure is associated with this ATP and peptide binding cycle (Zhu et al, 1996, Science 272:1606-14).

In addition to the classical heat shock and stress responsive proteins, the Hsp60, Hsp70 and Hsp90 families are also composed of proteins that are related to the HSPs in sequence, having greater than 35% amino acid identity, but whose expression levels are not altered by heat shock or stress.

Heat shock proteins are among the most highly conserved proteins in existence. For example, DnaK, the Hsp70 from E. coli has about 50% amino acid sequence identity with Hsp70 proteins from excoriates (Bardwell *et al.*, 1984, Proc. Natl. Acad. Sci. 81:848-852). The Hsp60 and Hsp90 families also show similarly high levels of intra-family conservation (Hickey *et al.*, 1989, Mol. Cell. Biol. 9:2615-2626; Jindal, 1989, Mol. Cell. Biol. 9:2279-2283).

2.2. Immunogenicity of Heat Shock/Stress Proteins

Srivastava *et al.* demonstrated immune response to methylcholanthrene-induced sarcomas of inbred mice (1988, Immunol. Today 9:78-83). In these studies, it was found that the molecules responsible for the individually distinct immunogenicity of these tumors were identified as cell-surface glycoproteins of 96kDa (gp96) and intracellular proteins of 84 to 86kDa (Srivastava *et al.*, 1986, Proc. Natl. Acad. Sci. USA 83:3407-3411; Ullrich, S.J. *et al.*, 1986, Proc. Natl. Acad. Sci. USA 83:3121-3125). Immunization of mice with gp96 or p84/86 isolated from a particular tumor rendered the mice immune to that particular tumor, but not to antigenically distinct tumors. Isolation and characterization of genes encoding gp96 and p84/86 revealed significant homology between them, and showed

that gp96 and p84/86 were, respectively, the endoplasmic reticular and cytosolic counterparts of the same heat shock proteins (Srivastava *et al.*, 1988, Immunogenetics 28:205-207; Srivastava *et al.*, 1991, Curr. Top. Microbiol. Immunol. 167:109-123). Further, Hsp70 was shown to elicit immunity to the tumor from which it was isolated but not to antigenically distinct tumors. However, Hsp70 depleted of peptides was found to lose its immunogenic activity (Udono and Srivastava, 1993, J. Exp. Med. 178:1391-1396). These observations suggested that the heat shock proteins are not immunogenic per se, but form noncovalent complexes with antigenic peptides, and the complexes can elicit specific immunity to the antigenic peptides (Srivastava, 1993, Adv. Cancer Res. 62:153-177; Udono *et al.*, 1994, J. Immunol., 152:5398-5403; Suto *et al.*, 1995, Science, 269:1585-1588).

The use of noncovalent complexes of stress proteins and peptides, purified from cancer cells, for the treatment and prevention of cancer, as well as the use of such complexes in combination with adoptive immunotherapy, has been described (see U.S. Patent No. 5,750,199; U.S. Patent No. 5,830,464; Patent Cooperation Treaty ("PCT") publications WO 96/10411, dated April 11, 1996; and WO 97/10001, dated March 20, 1997; each of which is incorporated by reference herein in its entirety. The purification of stress protein-peptide complexes from cell lysates has been described previously; stress protein-peptide complexes can be isolated from pathogen-infected cells and used for the treatment and prevention of infection caused by pathogens, such as viruses and other intracellular pathogens, including bacteria, protozoa, fungi and parasites (see PCT publication WO 95/24923, dated September 21, 1995).

Immunogenic stress protein-peptide complexes can also be prepared by *in vitro* complexing of stress protein and antigenic peptides, and the uses of such complexes for the treatment and prevention of infectious diseases and cancer has been described in PCT publication WO 97/10000, dated March 20, 1997. The use of heat shock proteins in combination with a defined antigen for the treatment of infectious diseases and cancer have also been described in PCT publication WO 97/06821, dated February 27, 1997. The administration of expressible polynucleotides encoding eukaryotic heat shock proteins to mammalian cells for stimulating an immune response, and for treatment of infectious diseases and cancer has been described in PCT publications, WO 97/06685 and WO 97/06828, both dated February 27, 1997. The use of stress protein-peptide complexes for sensitizing antigen presenting cells *in vitro* for use in adoptive immunotherapy is described in PCT publication WO 97/10002, dated March 20, 1997.

These references, however, have not identified portions of HSPs that could be effective in the treatment of infectious disease and cancer. Methods for identifying and producing such HPBFs would facilitate more efficient methods for producing HSPs and HSP–peptide complexes.

Citation or discussion of a reference herein shall not be construed as an admission that such is prior art to the present invention.

3. SUMMARY OF INVENTION

The present invention relates to pharmaceutical compositions comprising peptide-binding fragments of heat shock proteins (HSPs) and complexes of peptide-binding fragments of HSPs in noncovalent association with antigenic molecules. The invention further relates to methods for the use of such pharmaceutical compositions as immunotherapeutic agents for the prevention and treatment of infectious diseases and cancer.

In one embodiment, the invention provides a pharmaceutical composition comprising an amount of a molecular complex effective for treatment or prevention of an infectious disease or cancer, said molecular complex comprising a heat shock protein fragment noncovalently associated with an antigenic molecule and a pharmaceutically acceptable carrier, said heat shock protein fragment comprising that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400, and wherein said antigenic molecule displays the antigenicity of an antigen of an infectious agent or of a cancer cell. In specific embodiment, the heat shock protein of such a pharmaceutical composition is Hsp70, Hsp90, gp96, calreticulin, or PDI. In another embodiment, said heat shock protein fragment lacks one or more other domains of the heat shock protein.

In another embodiment, the invention provides a recombinant cell infected with a pathogen and transformed with a nucleic acid comprising a nucleotide sequence that (i) is operably linked to a promoter, and (ii) encodes a heat shock protein fragment comprising a peptide-binding domain that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400, which heat shock protein fragment noncovalently associates with an antigenic molecule when said antigenic molecule is present, to form a complex that in sufficient amount is capable of eliciting an immune response to the antigenic molecule. In one embodiment, said heat shock protein fragment lacks one or more other domains of the heat shock protein. In a specific embodiment, such a recombinant cell is a human cell.

The invention further provides a recombinant cancer cell transformed with a nucleic acid comprising a nucleotide sequence that (i) is operably linked to a promoter, and (ii) encodes a heat shock protein fragment comprising a peptide-binding domain, which heat shock protein fragment noncovalently associates with an antigenic molecule when said antigenic molecule is present to form a complex that in sufficient amount is capable of eliciting an immune response to the antigenic molecule. In one embodiment, said heat shock protein fragment lacks one or more other domains of the heat shock protein. In a specific embodiment, the cancer cell is a human cell.

In another embodiment, the invention provides a recombinant cell transformed with (i) a first nucleic acid comprising a first nucleotide sequence that is operably linked to a first promoter and that encodes a heat shock protein fragment comprising a peptide-binding domain that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400, and (ii) a second nucleic acid comprising a second nucleotide sequence that is operably linked to a second promoter and encodes an antigenic molecule, such that the heat shock protein fragment and the antigenic molecule are expressed within the cell and noncovalently associate with each other to form a complex that in sufficient amount is capable of eliciting an immune response to the antigenic molecule.

The invention further provides pharmaceutical compositions comprising recombinant cells, as described above, and a pharmaceutically acceptable carrier.

The invention further encompasses methods for preparation of noncovalent complexes of HSP peptide-binding fragments and antigenic peptide molecules. In one embodiment, the invention provides a method for preparing a complex of a heat shock protein peptide-binding fragment noncovalently associated with a peptide, said heat shock protein peptide-binding fragment comprising a peptide-binding domain that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400, comprising: a) culturing cells, transformed with a nucleic acid comprising a nucleotide sequence encoding the heat shock protein fragment and operably linked to a promoter, under conditions such that the heat shock protein fragment is expressed by the cells and associates with peptides of the cells; and b) recovering a population of complexes of the heat shock protein fragment noncovalently associated with peptides from the host cell. In one embodiment, the heat shock protein fragment of said method lacks one or more other heat shock protein domains.

The invention further provides a method for preparing a heat shock protein fragment noncovalently associated with peptides derived from one or more antigens of an infectious agent, said heat shock protein peptide-binding fragment comprising a peptide-binding domain that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400, comprising: a) culturing infected cells transformed with a nucleic acid comprising a nucleotide sequence encoding the heat shock protein peptide-binding fragment and operably linked to a promoter under conditions such that the heat shock protein peptide-binding fragment is expressed by the cells and associates with peptides of the cells; and b) recovering a population of complexes of the heat shock protein peptide-binding fragment and peptides derived from the infectious antigen.

In another embodiment, the invention further provides a method for preparing a complex of a heat shock protein fragment noncovalently associated with a peptide, said heat shock protein fragment comprising a peptide-binding domain that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400, said method comprising digesting a preparation of heat shock proteins noncovalently associated with peptides with a protease under conditions and for a length of time sufficient for the formation of fragments of the heat shock protein noncovalently associated with peptides.

In another embodiment, the invention further provides a method for preparing a complex of a heat shock protein fragment noncovalently associated with a peptide, said heat shock protein fragment comprising a peptide-binding domain that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400, said method comprising: a) digesting a preparation of heat shock proteins with a protease under conditions and for a length of time sufficient for the formation of fragments of the heat shock protein; and b) contacting the fragments with peptides under conditions and for a length of time sufficient for the formation of complexes of heat shock protein peptide-binding fragments noncovalently associated with peptides.

In specific embodiments, the methods for preparation of such HPBF-peptide complexes further comprise purifying the complexes. In another specific embodiment, the methods further comprise purifying the complexes by affinity chromatography.

The invention further provides a method for preparing *in vitro* complexes of heat shock protein peptide-binding fragments noncovalently associated with one or more antigenic molecules, said heat shock protein peptide-binding fragment comprising a peptide-binding domain that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400, said method comprising incubating a heat shock protein fragment and one or more antigenic molecules under conditions and for a length of time sufficient for the formation of the complexes. In one embodiment of this method, one or more antigenic molecules is a population of peptides from an infected cell or a cancer cell. In another embodiment of this method, one or more antigenic molecules display(s) the antigenicity of an antigen of an infectious agent or a cancer cell.

The invention further encompasses the use of heat shock protein peptide-binding fragments in methods for eliciting an immune response. In various embodiments, using *in vivo* and *in vitro* techniques, noncovalent complexes of heat shock protein peptide-binding fragments with antigenic molecules are produced. Such complexes may then be used to elicit an immune response by administering an effective amount of complex of HSP peptide-binding fragment bound to an antigenic molecule.

In one embodiment, the invention provides a method of eliciting an immune response against an antigen in an individual comprising administering to the individual an immunogenic complex of a heat shock protein fragment noncovalently associated with a first antigenic molecule displaying antigenicity of the antigen, said heat shock protein fragment comprising a peptide-binding domain that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400. In one embodiment, heat shock protein fragment of said method lacks one or more other heat shock protein domains. In another embodiment, the invention provides the above-described method, further comprising, before, concurrently, or after administration of the immunogenic complex, administering to the individual a composition comprising antigen presenting cells sensitized *in vitro* with a sensitizing amount of a second immunogenic complex consisting essentially of a heat shock protein, or fragment thereof,

noncovalently bound to a second antigenic molecule, in which said second antigenic molecule shares at least one antigenic determinant with the first antigenic molecule.

The invention further encompasses the use of heat shock protein peptide-binding fragments in methods for immunotherapy for treatment and prevention of infectious diseases. In various embodiments, heat shock protein peptide-binding fragments are used in methods for preventing or treating an infectious disease in an individual having an infectious disease, or in whom prevention of an infectious disease is desired. In various embodiments the infectious disease is caused by an infectious agent selected from the group consisting of viruses, bacteria, fungi, and parasites.

In one embodiment, a method is provided comprising administering to the individual an immunogenic complex of a heat shock protein peptide-binding fragment noncovalently associated with a first antigenic molecule, said heat shock protein peptide-binding fragment comprising a peptide-binding domain that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400, wherein the first antigenic molecule displays the antigenicity of an antigen of an infectious agent of the infectious disease. In one embodiment, the heat shock protein fragment of said method lacks one or more other heat shock protein domains. In another embodiment, the method further comprises, before, concurrently or after administration of the immunogenic complex, administering to the individual a composition comprising antigen presenting cells sensitized *in vitro* with a sensitizing amount of a second complex of a heat shock protein or fragment thereof noncovalently bound to a second antigenic molecule, said second antigenic molecule sharing at least one antigenic determinant with the first antigenic molecule.

In another embodiment, the invention further provides a method of treating or preventing an infectious disease in a subject having an infectious disease or in whom prevention of an infectious disease is desired comprising: a) culturing an infected cell transformed with a nucleic acid comprising a nucleotide sequence encoding a heat shock protein peptide-binding fragment, said infected cell displaying the antigenicity of an antigen of an infectious agent of the infectious disease, said heat shock protein peptide-binding fragment comprising a peptide-binding domain that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400, said nucleotide sequence being operably linked to a promoter, under conditions such that the fragment is expressed by the infected cells and associates with

peptides of the cell; b) recovering complexes of the heat shock protein fragments noncovalently associated with peptides from the infected cell; and c) administering to the subject an amount of the recovered complexes effective to treat or prevent the infectious disease. In another embodiment, the method further comprises prior to step (a) the step of obtaining infected cells from the subject and transforming the infected cells with the nucleic acid. In another embodiment, the method further comprises prior to step (a) the step of obtaining the infected cell from one or more individuals and transforming the infected cells with the nucleic acid, said one or more individuals being different from the subject and having the same type of infectious disease as the subject. In one embodiment, the heat shock protein fragment of said method lacks one or more other heat shock protein domains.

The invention further encompasses the use of heat shock protein peptide-binding fragments in methods for immunotherapy for treatment and prevention of cancer. In various embodiments, heat shock protein peptide-binding fragments are used in methods to prevent or inhibit growth of a tumor or treat an individual with cancer. In one embodiment a method is provided for treating or preventing cancer in an individual having a type of cancer or in whom prevention of a type of cancer is desired comprising administering to the individual an immunogenic complex of a heat shock protein fragment noncovalently associated with a first antigenic molecule, said heat shock protein comprising a peptide-binding domain that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400, wherein either (a) the first antigenic molecule displays antigenicity of said type of cancer or a metastasis thereof; or (b) the complex is obtained by recovering complexes from said type of cancer cells or a metastasis thereof that recombinantly express the heat shock protein fragment. In another embodiment, this method further comprises, before, concurrently or after administration of the immunogenic complex, administering to the individual a composition comprising antigen presenting cells sensitized *in vitro* with a sensitizing amount of a second complex of a heat shock protein or fragment thereof noncovalently bound to a second antigenic molecule, said second antigenic molecule sharing at least one antigenic determinant with the first antigenic molecule.

In another embodiment, the invention provides a method of treating or preventing cancer in a subject having a type of cancer or in whom prevention of a type of cancer is desired comprising: a) culturing a cancer cell transformed with a nucleic acid comprising a nucleotide sequence encoding a heat shock protein fragment comprising a peptide-binding domain that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is

contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400, said nucleotide sequence being operably linked to a promoter, under conditions such that the heat shock protein peptide-binding fragment is expressed by the cancer cells and associates with peptides of the cell; b) recovering complexes of the heat shock protein fragments noncovalently associated with peptides from the cancer cell; c) administering to the subject an amount of the recovered complexes effective to treat or prevent cancer. In another embodiment, the method further comprises prior to step (a) the step of obtaining cancer cells from the subject and transforming the cancer cells with the nucleic acid. In yet another embodiment, the method further comprises prior to step (a) the step of obtaining cancer cells from one or more individuals and transforming the cancer cells with the nucleic acid, said one or more individuals being different from the subject and having the same type of cancer as the subject.

In various embodiments of the invention such methods, specific Hsp70 family protein peptide-binding fragments are utilized with such methods. In a specific embodiment, the peptide-binding fragment is a fragment of 100 to 200 contiguous amino acids, comprising amino acid residues from the following positions of the amino acid sequence as shown in FIG. 1B (SEQ ID NO:2): 413 to 638, or 424 to 464. In another specific embodiment, the peptide-binding fragment is a fragment of 100 to 200 contiguous amino acids, comprising amino acid residues from position 391 to 615, or from position 400 to 440 of the amino acid sequence as shown in FIG. 1C (SEQ ID NO:3). In yet another embodiment, the heat shock protein is a human hsc70 peptide-binding fragment comprising 100 to 200 contiguous amino acids from positions 391 to 615, or from position 406 to 443 of the amino acid sequence as shown in FIG. 1D (SEQ ID NO:4).

In various embodiments of the invention such methods, specific Hsp90 family protein peptide-binding fragments are utilized with such methods. In a specific embodiment, the peptide-binding fragment is a fragment of 100 to 200 contiguous amino acids, comprising amino acid residues from position 5 to 232 of the amino acid sequence as shown in FIG. 2A (SEQ ID NO:5). In another embodiment, a human hsp84 peptide-binding fragment comprising 100 to 200 contiguous amino acids of positions 5 to 232 of the amino acid sequence as shown in FIG. 2B (SEQ ID NO:6) is utilized. In another embodiment, a gp96 peptide-binding fragment comprising 100 to 200 contiguous amino acids from position 5 to 232 of the amino acid sequence as shown in FIG. 2C (SEQ ID NO:7) is utilized. In yet another embodiment, a gp96 peptide-binding fragment comprising 100 to 200 contiguous amino acids from position 615 to 658 or 624 to 630 of the amino acid sequence as shown in FIG. 2C (SEQ ID NO:7) is utilized. In yet another embodiment, a human PDI protein

peptide-binding fragment comprising 100 to 200 contiguous amino acids from position 5 to 232 of the amino acid sequence as shown in FIG. 3 (SEQ ID NO:8) is utilized.

In yet other embodiments, human PDI protein peptide-binding fragments are utilized with the methods of the present invention. In a specific embodiment, a peptide-binding fragment comprising 75 to 139 contiguous amino acids from position 213 to 351 of the amino acid sequence as shown in FIG. 3 (SEQ ID NO:8) is used. In yet another embodiment, a human PDI protein peptide-binding fragment comprising 140 to 298 contiguous amino acids from position 204 to 491 of the amino acid sequence as shown in FIG. 3 (SEQ ID NO:8) is used.

4. BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 A-D. Amino acid sequence of human Hsp70 family proteins. Amino acid residues comprising the entire peptide-binding domain are underscored, amino acids of the β-helix motif are shown in bold, and amino acid residues of the central peptide-binding core are double underscored. **A.** Amino acid sequence of *E. coli* DnaK (SEQ ID NO:1). **B.** Amino acid sequence of the human Hsp70 family homologue, hBiP, also called grp78 (SEQ ID NO:2). **C.** Amino acid sequence of the inducible human Hsp70 family homologue, hsp71 (SEQ ID NO:3). **D.** Amino acid sequence of the constitutive human Hsp70 family homologue, hsc70 (SEQ ID NO:4).

FIG. 2 A-C. Amino acid sequences of human Hsp90 family proteins. Amino acid residues comprising the peptide-binding domain are underscored. **A.** Amino acid sequence of human Hsp 90 family homologue, hsp86, also called hsp90 (SEQ ID NO:5). **B.** Amino acid sequence of the human Hsp90 family homologue, hsp84, also called hsp90 (SEQ ID NO:6). **C.** Amino acid sequence of the inducible human Hsp90 family homologue gp96 (SEQ ID NO:7), showing two alternative peptide-binding domains. The amino acid residues of the central peptide-binding core of one of these peptide-binding domains are double underscored.

FIG. 3. Amino acid sequence of human PDI (SEQ ID NO:8).

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to compositions comprising noncovalent complexes of peptide-binding fragments of HSPs with antigenic molecules, and methods for their use as immunotherapeutic agents. The invention is based, in part, on the discovery by the inventors that a fragment containing the portion of an HSP molecule that binds peptides may be used to form complexes with antigenic peptides. Such peptide-binding HPBFs can be used in noncovalent association with antigenic molecules as immunotherapeutic agents. Such HSP-antigenic peptide complexes can be used in immunotherapy, for example, to treat or prevent an infectious disease or cancer.

The terms "HPBF" (HSP peptide-binding fragment), "HSP peptide-binding fragment", and "peptide-binding HSP fragment" are used interchangeably herein to refer to a polypeptide that comprises an HSP domain that is capable of becoming noncovalently associated with a peptide to form a complex, but that is not a full-length HSP. An HPBF of the invention is capable of eliciting an immune response, and comprises a peptide-binding domain that is contiguous on its N-terminal side with a variable number of amino acids that naturally flank the peptide-binding domain on the N-terminal side (herein termed "x" number of amino acids), and that is contiguous on the C-terminal side with a variable number of amino acids that naturally flank the peptide-binding domain on the C-terminal side (herein termed "y" number of amino acids), wherein x plus y is not more than 400 amino acids. As used herein, a number of "contiguous amino acids that naturally flank the peptide binding domain" refers to a number of contiguous amino acid sequences, adjacent to the peptide-binding domain, in either or both directions, which are also present in the native heat shock protein. Preferably, said fragment lacks one or more other heat shock protein domains.

Such an HPBF may be a polypeptide of less than 100 amino acids, or of 100 to 200 amino acids, 200 to 400 amino acids, or 400 to 800 amino acids. In the preferred embodiment, an HSP peptide-binding fragment is a polypeptide of approximately 100 to 200 amino acids. In various embodiments, x plus y is not more than 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 30, 380, 390, or 400 amino acids in length.

In one embodiment, for example, x is not more than 10, 12, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 30, 380, 390, or 400 amino acids in length. For example, x can be between 0 to 5 amino acids, 5 to 10 amino acids, 10 to 30 amino acids, 30 to 50 amino acids, 50 to 100

amino acids, 100 to 150 amino acids, 150 to 200 amino acids, 200 to 250 amino acids, 250 to 300 amino acids, 300 to 350 amino acids, or 350 to 400 amino acids in length.

In one embodiment, for example, y is not more than 10, 12, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 30, 380, 390, or 400 amino acids in length. For example, y can be between 0 to 5 amino acids, 5 to 10 amino acids, 10 to 30 amino acids, 30 to 50 amino acids, 50 to 100 amino acids, 100 to 150 amino acids, 150 to 200 amino acids, 200 to 250 amino acids, 250 to 300 amino acids, 300 to 350 amino acids, or 350 to 400 amino acids in length.

Non-limiting examples of other heat shock protein domains, one or more of which can be lacking in an HPBF, include: nucleotide-binding domains, such as an ATP-binding domain; domains comprising amino acids involved in ATP hydrolysis; oligomerization domains, such as a dimerization domain; domains encompassing chaperone binding sites; charged domains; middle domains; domains encompassing co-chaperone or cofactor binding sites, such as a domain involved in binding of HSP cofactors having tetratricopeptide repeat domains; hinged domains; linker domains; nuclear localization region; cytoplasmic localization region; and domains involved in protein-folding, provided that the lacking domain is not also required for binding peptide.

In addition, the polypeptide may comprise one or more other domains, and optionally lack all other sequences of the HSP from which the peptide-binding domain is derived. The additional domains of such chimeric HPBFs may be derived from another portion of an HSP molecule, or alternatively, from a heterologous protein. The heterologous protein may be a different heat shock protein or another type of heterologous protein. For example, chimeric HPBF molecules may comprise protein domains that assist in the expression, detection, or recovery of the chimeric HPBF, or in the induction of an immune response.

The invention comprises pharmaceutical compositions comprising HSP peptide-binding fragments in noncovalently bound complexes with antigenic molecules for use in eliciting an immune response for treatment of infectious diseases and cancer. Heat shock proteins useful for obtaining the peptide-binding HSP fragments of the present invention can be selected from among cellular proteins that satisfy any one of the following criteria: the intracellular concentration of such protein increases when a cell is exposed to a stressful stimulus; such protein can bind other proteins or peptides, and can release the bound proteins or peptides in the presence of adenosine triphosphate (ATP) or low pH; or such protein possesses at least 35% homology with any cellular protein having any of the above properties.

HPBFs and HPBF–peptide complexes comprising members of the five major families of HSPs, namely Hsp60, Hsp70, Hsp90, Hsp100, and sHsps, can be prepared by the practice of the present invention. HPBFs include, but are not limited to, the peptide-binding domain fragments of an Hsp70 family protein, such as hsp71, hsc70, BiP, and DnaK, peptide-binding domain fragments of an Hsp90 family protein, such as hsp90, hsp80, hsp81, hsp82, hsp83, hsp84, hsp85, hsp86, hsp100, frp94, endoplasmin, and gp96. In addition, calreticulin, PDI and other proteins in the endoplasmic reticulum that contain thioredoxin-like domain(s), such as but not limited to ERp72 and ERp61, are also encompassed. In various embodiments, the methods provided herein for treatment and prevention of infectious disease and cancer utilize HPBFs comprising a peptide-binding fragment of one type of HSP, or a mixture of peptide-binding fragments of two or more different HSP proteins.

HPBFs may also comprise other related members of the Hsp60, Hsp70, Hsp90, Hsp100, sHsps, calreticulin and PDI families, proeins that are related to HSPs in sequence, for example, having greater than 35% amino acid identity, but whose expression levels are not altered by stress. Therefore it is contemplated that the definition of heat shock or stress protein, as used herein, embraces other proteins, mutants, analogs, and variants thereof having at least 35% to 55%, preferably 55% to 75%, and most preferably 75% to 85% amino acid identity with members of these families whose expression levels in a cell are enhanced in response to a stressful stimulus.

The invention further comprises recombinant cells that express HPBF–peptide complexes. In a preferred embodiment, the HPBFs and that can be prepared by the present invention include peptide-binding fragments of Hsp70, Hsp90, gp96, protein disulfide isomerase (PDI), calreticulin, or sHSP, alone or in combination. Preferably, the HSPs are human HSPs. A preferred complex comprises a human fragment comprising the peptide-binding domain of Hsp60, Hsp70, Hsp90, calreticulin, PDI, or sHSP, noncovalently bound to a human protein antigen. Using the defined amino acid or cDNA sequences of peptide-binding domains of a given HSP one can make a genetic construct which is transfected into and expressed in a host cell. The genetically engineered host cells may contain one or more copies of a nucleic acid sequence comprising a sequence that encodes an HSP peptide-binding domain, operably associated with regulatory region(s) that drives the expression of the HSP nucleic acid sequence in the host cell. Any nucleic acid sequence encoding an HSP peptide-binding domain, including cDNA and genomic DNA, can be used to prepare the peptide-binding fragments of the invention. It is preferred that the recombinant HSP produced in the host cell or library cell is of the same species as the intended recipient of the immunogenic composition. Recombinant human HSP is most preferred.

In various embodiments, the invention provides methods for the use of HSP peptide-binding fragments in immunotherapy. An effective amount of complex of an HSP peptide-binding fragment bound to an antigenic molecule can be administered to a patient to elicit an immune response. The HSP peptide–binding fragments can further be used in adoptive immunotherapy treatment. Compositions of complexes of peptide-binding domain fragments of HSPs, noncovalently bound to antigenic molecules, alone or in combination with each other, are administered in combination with the administration of antigen presenting cells sensitized with an HSP-antigenic molecule noncovalent complex to augment the immune response to genotoxic and nongenotoxic factors, tumors and infectious agents. Such HPBF complexes are delivered in combination with immune cells or in combination with interferons, cytokines, etc.

5.1 HSP Peptide-Binding Domain Fragments

Heat shock/stress proteins are composed of structural domains that correspond to their various functional activities, including peptide binding and ATP binding and hydrolysis. In the Hsp70 and Hsp90 families, an ATP hydrolysis cycle regulates substrate binding. Fragments of HSPs lacking the ATP-binding domain can bind peptide substrate; the hydrolysis of ATP is required for release of the peptide and uptake of another substrate. Thus, a fragment of an HSP molecule can be isolated that lacks the ATP-binding domain yet retains the peptide-binding activity of the full-length molecule, and such an HPBF can be used in immunotherapeutic treatments against infectious disease and cancer. The peptide-binding domains of several HSPs of different families are described in detail below.

The peptide-binding domain of DnaK, the bacterial homologue of mammalian Hsp70, has been determined (Zhu *et al.*, 1996, Science 272:1606-1614). The peptide-binding domain, spanning amino-acid residues 388 to 606 of DnaK (SEQ ID NO:1), shown underlined in FIG. 1A, is composed of a β sandwich structure, and an adjacent extended structure of α helices, having overall dimensions of ~ 60 by 40 by 15 Å. The β-helix structure, comprising residues 392 to 501, is arranged in two sheets with 4 antiparallel β strands. Substrate binding is almost completely determined by substrate interactions of five amino acid residues at the core of the β-helix structure. This region is exceptionally well conserved in other members of the Hsp70 family, including human Hsc70 and human BiP, where the range of amino acid identity is 65 to 76 percent. The interaction of peptide and the β-helix motif is stabilized by a less well conserved neighboring α-helical region comprising residues 537 to 606.

In accordance with the present invention, HPBFs are constructed or prepared comprising amino acid residues of Hsp70 family peptide-binding domains. In one embodiment, for example, an HPBF comprises a fragment of about 100 contiguous amino acids of positions 388 to 606 of DnaK (SEQ ID NO:1), *i.e.*, the amino acids underscored in FIG 1A. Preferably, in another embodiment, such an HPBF comprises a fragment of about 100 contiguous amino acids of positions 393 to 500 of DnaK (SEQ ID NO:1), *i.e.*, the amino acids shown in bold in FIG 1A. More preferably, in yet another embodiment, such an HPBF comprises the central core of the peptide-binding domain a fragment, about 50 to100 contiguous amino acids of positions 399 to 439 of DnaK (SEQ ID NO:1), *i.e.*, the amino acids double-underscored in FIG 1A.

HPBFs comprising peptide-binding domains of human Hsp70 proteins that correspond to the DnaK peptide-binding domain are also encompassed by the present invention. For example, human Bip, otherwise known as grp78, is a human homologue of DnaK and a member of the Hsp70 family; the amino acid sequence of which is shown in FIG. 1B. In one embodiment, an HPBF comprises a fragment of about 50 to100 contiguous amino acids of positions 413 to 638 of human BiP (SEQ ID NO:2), *i.e.*, the amino acids underscored in FIG 1B. In a preferred embodiment, such an HPBF comprises a fragment of about 100 contiguous amino acids of positions 418 to 524 of human BiP (SEQ ID NO:2), *i.e.*, the amino acids shown in bold in FIG 1B. In yet a more preferred embodiment, an HPBF comprises about 50 to 100 contiguous amino acids of positions 424 to 464 of human BiP (SEQ ID NO:2), *i.e.*, the amino acids double-underscored in FIG 1B.

In another embodiment, an HPBF comprises a fragment of about 50 to100 contiguous amino acids of positions 391 to 615 of the inducible form of a human Hsp70 family protein, hsp71 (SEQ ID NO:3), *i.e.*, the amino acids underscored in FIG 1C. In a preferred embodiment, such an HPBF comprises a fragment of about 100 contiguous amino acids of positions 395 to 502 of human hsp71 (SEQ ID NO:3), *i.e.*, the amino acids shown in bold in FIG 1C. In a more preferred embodiment, an HPBF comprises about 50 to 100 contiguous amino acids of positions 400 to 440 of human hsp71 (SEQ ID NO:3), *i.e.*, the amino acids double-underscored in FIG 1C.

In another embodiment, an HPBF comprises a fragment of about 50 to100 contiguous amino acids of positions 391 to 615 of the constitutive form of a human Hsp70 family protein, hsc70 (SEQ ID NO:4), *i.e.*, the amino acids underscored in FIG 1D. In a preferred embodiment, such an HPBF comprises a fragment of about 100 contiguous amino acids of positions 395 to 504 of human hsp71 (SEQ ID NO:4), *i.e.*, the amino acids shown in bold in FIG 1D. In a more preferred embodiment, an HPBF comprises about 50 to 100 contiguous amino acids of positions 406 to 443 of human hsp71 (SEQ ID NO:4), *i.e.*, the amino acids double-underscored in FIG 1D.

In another embodiment, the Hsp70 fragment comprises a peptide-binding domain plus any other domain of interest, such as the ATP-binding domain. In addition, the invention encompasses the corresponding regions of other members of the highly conserved Hsp70 family of proteins. In addition, the invention encompasses chimeric proteins composed of Hsp70 peptide-binding domains linked to other proteins, or fragments or domains thereof.

The Hsp90 family of proteins are ubiquitous molecular chaperones which, in eukaryotes, are involved in protein folding of a broad range of important substrates, such as, for example, cellular proteins such as transcription factors, hormone receptors, tyrosine kinases, and anti-oncogenes. Hsp90s are dimeric proteins, having monomers of 80-90 kDa. Members of the Hsp 90 family are highly conserved, especially in their N-terminal and C-terminal regions, which constitute distinct functional domains (Scheibel and Buchner, 1997, in "Guidebook to Chaperones", Gething, M. J. (ed.), Oxford Univ. Press, Oxford, pp. 147-150). The N-terminal residues 5 to 232 contain a compact ligand-binding pocket comprising the peptide-binding domain, in addition to a nucleotide binding site, a geldanomycin binding site, and an interaction site for unfolded proteins (Stebbins *et al.*, 1997, 89: 239-250; Prodromou *et al.*, 1997, Nat. Struct. Biol. 4: 477-482; Prodromou *et al.*, 1997, Cell 90: 65-75; Wang *et al.*, 1993, J. Biol. Chem. 268: 26049-51). Adjacent to the N-terminal ligand-binding pocket, from about residues 210 to 272, is a highly charged linker domain, which modulates the activity of the N-terminal domain, by decreasing the affinity of peptide-bound N-terminal domain for binding of ATP and geldanamycin (Scheibel *et al.*, 1998, Proc. Natl. Acad. Sci. USA 95: 1495-1499). The C-terminal domain comprises the oligomerization domain (residues 676 to 719; Wearsch and Nicchitta, 1996, Biochemistry 35:16760-9), an ATP-independent chaperone site (residues 262 to 709; Scheibel *et al.*, 1998, Proc. Natl. Acad. Sci. USA 95: 1495-1499), and a TPR domain, which specifically binds Hsp90 cofactors having TPR (tetratricopeptide repeat) domains (residues 558 to 724; Young *et al.*, 1998, J. Biol. Chem. 273:18007-10; Young *et al.*, 1997, FEBS Lett. 418: 139-143; Carrello *et al.*, 1999, J. Biol. Chem., 274: 2682-9). The N-terminal and C-terminal domains are connected by a large middle domain, from about residues 294 to 600, which is highly conserved among Hsp90 family proteins. This region comprises a region involved in nuclear localization between amino acid residues 381 and 581, a cytoplasmic localization region between residues 287 and 728, and a glucocorticosteroid receptor interaction domain between residues 446 and 581 (Jibard *et al.*, 1999, Exp. Cell. Res., 15: 461-74). An alternative peptide-binding site of the Hsp90 family member, gp96, has been mapped to amino acid residues 615 through 658 of gp96, with a central peptide-binding core mapping to amino acid residues 624 through 630 of gp96 (Linderoth *et. al.*, 2000, J. Biol. Chem. 275: 5472-77).

Hsp90 peptide-binding fragments can be constructed or prepared that encompass the ligand domain, from about amino acid residues 5 to 272, or a peptide-binding fragment thereof. In another embodiment, an Hsp90 peptide-binding fragment can comprise amino acids 5 to 232, or a peptide-binding fragment thereof. In another embodiment, gp96 peptide-binding fragments can be constructed or prepared that encompasses amino acid residues 615 through 658 of gp96, or a peptide-binding fragment thereof. In another embodiment, an gp96 peptide-binding fragment can comprise amino acids 624 through 630 of gp96, or a peptide-binding fragment thereof (shown by the doubly underscored amino acids in FIG. 2C [SEQ ID NO:7]).

Peptide-binding fragments from members of the Hsp90 family used in accordance with the invention may include the substrate-binding domain either alone or together with additional domains, such as, for example, a second substrate-binding domain, the oligomerization domain, the C-terminal domain, the charged domain, the chaperone site, or the substrate-binding domain together with any other domain of interest, such as the charged domain or the nucleotide-binding site (but optionally not containing other parts of the molecule). In addition, the invention encompasses the corresponding regions of other members of the highly conserved Hsp90 family of proteins. In addition, the invention encompasses chimeric proteins composed of Hsp90 peptide-binding domains linked to other proteins, or fragments or domains thereof.

Protein disulfide isomerase (PDI) is an HSP of 491 amino acids, involved in folding of many disulfide-bonded proteins. Structure–function studies using PDI fragments have lead to a definition of PDI functional domains (Klappa *et al.*,1998, EMBO J., 17:927-935). These studies indicate that PDI contains various domains that contribute to peptide binding, unlike the Hsp90 and Hsp70 families of proteins, which appear to have discrete substrate binding domains. A PDI fragment that includes amino acid residues 213 to 351 is capable of binding short peptides (10-15 amino acids). A larger fragment, including amino acids 204 to 491, is able to bind of larger polypeptides of 25 to 50 amino acids. An even larger PDI fragment, including amino acids residues 1 to 370 is required for more complex substrates, such as larger polypeptides.

In a preferred embodiment, PDI peptide-binding fragments are constructed for use in accordance with the present invention that encompass the minimal peptide-binding domain (amino acids 213 to 351), capable of binding small peptides (10-15 amino acids). In an alternative embodiment, larger PDI peptide-binding fragments can be constructed that encompass the peptide-binding domain (amino acids 204 to 491) capable of binding larger polypeptides (for example, 28 amino acids). In yet another embodiment, a large PDI peptide-binding fragment (amino acids 1 to 370), capable of binding complex polypeptides (such as 'scrambled' RNase A, a natural substrate of PDI). Combinations of

any of these domains together with each other or with other PDI domains, such as the ATP-binding domain, or combinations of any of these domains together with domains of heterologous proteins, for example other HSP domains, are also within the scope of the invention. An entire domain or a fragment thereof can be used to prepare such constructs.

Small HSPs (sHSPs) range in size from 12K to 42K and form large multimeric structures of 200K to 400K. sHSPs have a wide range of cellular functions, including thermoprotection *in vivo* and chaperoning *in vitro*. The crystal structure of an sHSP (Kim *et al.*, 1998, Nature 394:595-599) shows that 24 monomeric subunits of sHSPs form a hollow spherical complex of octahedral symmetry, the N-terminal residues of which seem to be important for chaperone activity. The entire domain encompassing such N-terminal residues, or a fragment thereof, can be used to construct peptide-binding fragments for use in the present invention.

While the precise boundaries of the peptide-binding domain of the above-mentioned HSPs have been determined, others can be determined by sequence comparison to other members of the analogous HSP family. The high conservation of the substrate-binding amino acids and domains within HSP protein families makes it possible to predict the corresponding peptide-binding region in a given heat shock protein family member. Even HSPs whose domain structure have not yet been precisely mapped are within the scope of the present invention. For example, the endoplasmic reticular Hsp90 family protein, gp96, is known to contain several ATP-binding regions and presumably possesses a peptide-binding domain since gp96 is known to bind peptides.

As discussed above, the details of the peptide-binding domains of several HSPs are known. However, it is not necessary to know the precise boundaries of the peptide- binding domain of an HSP to use such an HPBF comprising a peptide-binding domain with the methods of the invention. Recombinant DNA techniques can be readily utilized to generate recombinant fragments of HSP genes, and standard recombinant techniques can be used to express such HSP gene fragments. Such recombinant HPBFs can be assayed for peptide binding activity (see for example, Klappa *et al.*,1998, EMBO J., 17:927-935) for their ability to elicit an immune response (as described in Section 5.4.1, herein). Such peptide-binding domains can be purified, or used in a non-purified form. Alternatively, HPBFs comprising peptide binding domains can be generated from native HSPs by protease cleavage of native HSPs or native HSP-peptide complexes, as described in detail below in Section 5.2.3.

Alternatively, the peptide-binding domain of an HSP may be predicted from its primary sequence, by sequence comparison to known HSPs and other peptide-binding proteins. HSPs share similar structural properties with peptide-binding domains from other peptide-binding proteins, such as class I and class II major histocampatibility (MHC)

complex antigen presenting molecules, SH2 and SH3 domains, protein kinases and phosphatase, proteases, antibodies, in that they bind peptide segments in extended conformations. Thus, one can search protein and nucleotide sequence databases to identify sequences with various degrees of similarities to these conserved peptide-binding motifs, using search programs such as FASTA and BLAST which rank the similar sequences by alignment scores and statistics.

To determine a region of identity between two amino acid sequences or nucleic acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = # of identical overlapping positions/total # of positions x 100%). In one embodiment, the two sequences are the same length.

The determination of percent identity between two sequences can also be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul, 1990, Proc. Natl. Acad. Sci. USA 87:2264-2268, modified as in Karlin and Altschul, 1993, Proc. Natl. Acad. Sci. USA 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, *et al.*, 1990, J. Mol. Biol. 215:403-410. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to a protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*, 1997, Nucleic Acids Res.25:3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships between molecules (Altschul *et al.*, 1997, *supra*). When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used (see http://www.ncbi.nlm.nih.gov). Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, 1988, CABIOS 4:11-17. Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When

utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used. The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, typically only exact matches are counted.

Functionally active derivatives and analogs of HSP peptide-binding domains are also within the scope of the present invention. In one embodiment, for example, Hsp70 peptide-binding domain derivatives and analogs can be designed. By computer modeling the three dimensional structure of the Hsp70 peptide-binding site, Hsp70 variants can be designed in which amino acid residues not involved in peptide binding or structurally important determinants may be substituted for the wild-type residues.

In another embodiment, where the three dimensional structure of the peptide-binding site of an HSP is unknown, its structure can be determined. This can be done using methods known to those of skill in the art, including X-ray crystallography. Alternatively, solid or liquid phase NMR can be used to determine certain intra-molecular distances. Any other experimental method of structure determination can be used to obtain partial or complete molecular structures. The molecular structures may be measured with a complexed peptide or other ligand, natural or artificial.

If an incomplete or insufficiently accurate structure is determined, the methods of computer based numerical modeling can be used to complete the structure or improve its accuracy. Any recognized modeling method may be used, including parameterized models specific to particular biopolymers such as proteins or nucleic acids, molecular dynamics models based on computing molecular motions, statistical mechanics models based on thermal ensembles, or combined models. For most types of models, standard molecular force fields, representing the forces between constituent atoms and groups, are necessary, and can be selected from force fields known in physical chemistry. The incomplete or less accurate experimental structures can serve as constraints on the complete and more accurate structures computed by these modeling methods.

Examples of molecular modeling systems are the CHARMm and QUANTA programs (Polygen Corporation, Waltham, MA). CHARMm performs the energy minimization and molecular dynamics functions. QUANTA performs the construction, graphic modeling and analysis of molecular structure. QUANTA allows interactive construction, modification, visualization, and analysis of the behavior of molecules with each other.

5.2 Preparation of HSP Peptide-Binding Fragments

The HSP peptide-binding fragment polypeptide of the invention may be produced by recombinant DNA techniques, synthetic methods, or by enzymatic or chemical cleavage of native HSPs. Described herein are methods for producing such HSP peptide-binding fragments.

5.2.1. Production of Recombinant HSP Peptide-Binding Fragments

5.2.1.1 Isolation of HSP Gene Sequences

In various aspects, the invention relates to amino acid sequences of peptide-binding fragments of HSPs. Nucleic acids encoding the HPBFs are provided, as well as nucleic acids complementary to and capable of hybridizing to such nucleic acids.

Any eukaryotic cell may serve as the nucleic acid source for obtaining the coding region of an *hsp* gene. Nucleic acid sequences encoding HSPs can be isolated from vertebrate, mammalian, as well as primate sources, including humans.

Amino acid sequences and nucleotide sequences of naturally occurring HSPs are generally available in sequence databases, such as GenBank. Computer programs, such as Entrez, can be used to browse the database, and retrieve any amino acid sequence and genetic sequence data of interest by accession number. These databases can also be searched to identify sequences with various degrees of similarities to a query sequence using programs, such as FASTA and BLAST, which rank the similar sequences by alignment scores and statistics. Such nucleotide sequences of non-limiting examples of HSPs that can be used for preparation of the HSP peptide-binding fragments of the invention are as follows: human Hsp70, Genbank Accession No. NM_005345, Sargent *et al.*, 1989, Proc. Natl. Acad. Sci. U.S.A., 86:1968-1972; human Hsp90, Genbank Accession No. X15183, Yamazaki *et al.*, Nucl. Acids Res. 17:7108; human gp96: Genbank Accession No. X15187, Maki *et al.*, 1990, Proc. Natl. Acad Sci., 87: 5658-5562; human BiP: Genbank Accession No. M19645; Ting *et al.*, 1988, DNA 7: 275-286; human Hsp27, Genbank Accession No. M24743; Hickey *et al.*, 1986, Nucleic Acids Res. 14:4127-45; mouse Hsp70: Genbank Accession No. M35021, Hunt *et al.*, 1990, Gene, 87:199-204; mouse gp96: Genbank Accession No. M16370, Srivastava *et al.*, 1987, Proc. Natl. Acad. Sci., 85:3807-3811; and mouse BiP: Genbank Accession No. U16277, Haas *et al.*, 1988, Proc. Natl. Acad. Sci. U.S.A., 85: 2250-2254. Due to the degeneracy of the genetic code, the term "HSP gene", as used herein, refers not only to the naturally occurring nucleotide sequence but also encompasses all the other degenerate DNA sequences that encode the HSP.

The DNA may be obtained by standard procedures known in the art by DNA amplification or molecular cloning directly from a tissue, cell culture, or cloned DNA (*e.g.*, a DNA "library"). Clones derived from genomic DNA may contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will contain only

exon sequences. Whatever the source, the *hsp* gene should be cloned into a suitable vector for propagation of the gene.

In a preferred embodiment, DNA can be amplified from genomic or cDNA by polymerase chain reaction (PCR) amplification using primers designed from the known sequence of a related or homologous HSP. PCR is used to amplify the desired sequence in DNA clone or a genomic or cDNA library, prior to selection. PCR can be carried out, *e.g.*, by use of a thermal cycler and Taq polymerase (Gene Amp®). The DNA being amplified can include cDNA or genomic DNA from any species. Oligonucleotide primers representing known nucleic acid sequences of related HSPs can be used as primers in PCR. In a preferred aspect, the oligonucleotide primers represent at least part of the *hsp* gene that is highly conserved between HSPs of different species. One can choose to synthesize several different degenerate primers, for use in the PCR reactions. It is also possible to vary the stringency of hybridization conditions used in priming the PCR reactions, to allow for greater or lesser degrees of nucleotide sequence similarity between the known HSP nucleotide sequence and the nucleic acid homologue being isolated. For cross species hybridization, low stringency conditions are preferred. For same species hybridization, moderately stringent conditions are preferred. After successful amplification, the sequence encoding an HSP may be cloned and sequenced. If the size of the coding region of the *hsp* gene being amplified is too large to be amplified in a single PCR, several PCR covering the entire gene, preferably with overlapping regions, may be carried out, and the products of the PCR ligated together to form the entire coding sequence. Alternatively, if a segment of an *hsp* gene is amplified, that segment may be cloned, and utilized as a probe to isolate a complete cDNA or genomic clone.

In another embodiment, for the molecular cloning of an hsp gene from genomic DNA, DNA fragments are generated to form a genomic library. Since some of the sequences encoding related HSPs are available and can be purified and labeled, the cloned DNA fragments in the genomic DNA library may be screened by nucleic acid hybridization to the labeled probe (Benton and Davis, 1977, Science 196:180; Grunstein and Hogness, 1975, Proc. Natl. Acad. Sci. U.S.A. 72:3961). Those DNA fragments with substantial homology to the probe will hybridize. It is also possible to identify the appropriate fragment by restriction enzyme digestion(s) and comparison of fragment sizes with those expected according to a known restriction map if such is available.

Alternatives to isolating the *hsp* genomic DNA include, but are not limited to, chemically synthesizing the gene sequence itself from a known sequence or making cDNA to the mRNA which encodes the HSP. For example, RNA for cDNA cloning of the *hsp* gene can be isolated from cells which express the HSP. A cDNA library may be generated by methods known in the art and screened by methods, such as those disclosed for

screening a genomic DNA library. If an antibody to the HSP is available, the HSP may be identified by binding of labeled antibody to the putatively HSP synthesizing clones.

Other specific embodiments for the cloning of a nucleotide sequence encoding an HSP, are presented as examples but not by way of limitation, as follows:

In a specific embodiment, nucleotide sequences encoding heat shock protein within a family can be identified and obtained by hybridization with a probe comprising nucleotide sequence encoding an HSP under conditions of low to medium stringency.

By way of example and not limitation, procedures using such conditions of low stringency are as follows (see also Shilo and Weinberg, 1981, Proc. Natl. Acad. Sci. USA 78:6789-6792). Filters containing DNA are pretreated for 6 h at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution with the following modifications: 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml salmon sperm DNA, 10% (wt/vol) dextran sulfate, and 5-20 X 10^6 cpm ^{32}P-labeled probe is used. Filters are incubated in hybridization mixture for 18-20 h at 40°C, and then washed for 1.5 h at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS. The wash solution is replaced with fresh solution and incubated an additional 1.5 h at 60°C. Filters are blotted dry and exposed for autoradiography. If necessary, filters are washed for a third time at 65-68°C and reexposed to film. Other conditions of low stringency which may be used are well known in the art (*e.g.*, as employed for cross-species hybridizations).

An HSP gene fragment containing the peptide-binding domain can be inserted into an appropriate cloning vector and introduced into host cells so that many copies of the gene sequence are generated. A large number of vector-host systems known in the art may be used such as, but not limited to, bacteriophages such as lambda derivatives, or plasmids such as pBR322 or pUC plasmid derivatives or the Bluescript vector (Stratagene).

Any technique for mutagenesis known in the art can be used to modify individual nucleotides in a DNA sequence, for purpose of making amino acid substitution(s) in the expressed peptide sequence, or for creating/deleting restriction sites to facilitate further manipulations. Such techniques include but are not limited to, chemical mutagenesis, *in vitro* site-directed mutagenesis (Hutchinson, C., *et al.*, 1978, J. Biol. Chem 253:6551), oligonucleotide-directed mutagenesis (Smith, 1985, Ann. Rev. Genet. 19:423-463; Hill *et al.*, 1987, Methods Enzymol. 155:558-568), PCR-based overlap extension (Ho *et al.*, 1989, Gene 77:51-59), PCR-based megaprimer mutagenesis (Sarkar *et al.*, 1990, Biotechniques, 8:404-407), etc. Modifications can be confirmed by double stranded dideoxy DNA sequencing.

The polymerase chain reaction (PCR) is commonly used for obtaining genes or gene fragments of interest. For example, a nucleotide sequence encoding an HSP peptide-binding fragment of any desired length can be generated using PCR primers that flank the nucleotide sequence encoding the peptide-binding domain. Alternatively, an *hsp* gene sequence can be cleaved at appropriate sites with restriction endonuclease(s) if such sites are available, releasing a fragment of DNA encoding the peptide-binding domain. If convenient restriction sites are not available, they may be created in the appropriate positions by site-directed mutagenesis and/or DNA amplification methods known in the art (see, for example, Shankarappa *et al.*, 1992, PCR Method Appl. 1:277-278). The DNA fragment that encodes the HSP peptide-binding domain is then isolated, and ligated into an appropriate expression vector, care being taken to ensure that the proper translation reading frame is maintained.

The HSP peptide-binding fragments of the invention may be expressed as fusion proteins to facilitate recovery and purification from the cells in which they are expressed. For example, the HPBF may contain a signal sequence leader peptide to direct its translocation across the ER membrane for secretion into culture medium. Further, the HPBF may contain an affinity label, such as a affinity label, fused to any portion of the HPBF not involved in binding antigenic peptide, such as for example, the carboxyl terminal. The affinity label can be used to facilitate purification of the protein, by binding to an affinity partner molecule.

Various methods for production of such fusion proteins are well known in the art. The manipulations which result in their production can occur at the gene or protein level, preferably at the gene level. For example, the cloned coding region of an HPBF may be modified by any of numerous recombinant DNA methods known in the art (Sambrook *et al.*, 1990, Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; Ausubel *et al.*, in Chapter 8 of Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, New York). It will be apparent from the following discussion that substitutions, deletions, insertions, or any combination thereof are introduced or combined to arrive at a final nucleotide sequence encoding an HSP peptide-binding fragment.

In various embodiments, fusion proteins comprising the HSP peptide-binding fragment may be made using recombinant DNA techniques. For example, a recombinant gene encoding an HSP peptide-binding fragment may be constructed by introducing an *hsp* gene fragment in the proper reading frame into a vector containing the sequence of an affinity label, such that the HPBF is expressed as a peptide-tagged fusion protein. Affinity labels, which may be recognized by specific binding partners, may be used for affinity purification of the HPBF.

In a preferred embodiment, the affinity label is fused at its amino terminal to the carboxyl terminal of the HSP. The precise site at which the fusion is made in the carboxyl terminal is not critical. The optimal site can be determined by routine experimentation. The immunogenicities of the HSP peptide-binding fragment can be tested by methods described in Section 5.4.1.

A variety of affinity labels known in the art may be used, such as, but not limited to, the immunoglobulin constant regions, polyhistidine sequence (Petty, 1996, Metal-chelate affinity chromatography, in Current Protocols in Molecular Biology, Vol. 2, Ed. Ausubel *et al.*, Greene Publish. Assoc. & Wiley Interscience), glutathione S-transferase (GST; Smith, 1993, Methods Mol. Cell Bio. 4:220-229), the *E. coli* maltose binding protein (Guan *et al.*, 1987, Gene 67:21-30), and various cellulose binding domains (U.S. Patent Nos. 5,496,934; 5,202,247; 5,137,819; Tomme *et al.*, 1994, Protein Eng. 7:117-123), etc. Other affinity labels may impart fluorescent properties to an HPBF, *e.g.*, portions of green fluorescent protein and the like. Other possible affinity labels are short amino acid sequences to which monoclonal antibodies are available, such as but not limited to the following well known examples, the FLAG epitope, the myc epitope at amino acids 408-439, the influenza virus hemagglutinin (HA) epitope. Other affinity labels are recognized by specific binding partners and thus facilitate isolation by affinity binding to the binding partner which can be immobilized onto a solid support. Some affinity labels may afford the HPBF novel structural properties, such as the ability to form multimers. Dimerization of an HPBF with a bound peptide may increase avidity of interaction between the HPBF and its partner in the course of antigen presentation. These affinity labels are usually derived from proteins that normally exist as homopolymers. Affinity labels such as the extracellular domains of CD8 (Shiue *et al.*, 1988, J. Exp. Med. 168:1993-2005), or CD28 (Lee *et al.*, 1990, J. Immunol. 145:344-352), or portions of the immunoglobulin molecule containing sites for interchain disulfide bonds, could lead to the formation of multimers. As will be appreciated by those skilled in the art, many methods can be used to obtain the coding region of the above-mentioned affinity labels, including but not limited to, DNA cloning, DNA amplification, and synthetic methods. Some of the affinity labels and reagents for their detection and isolation are available commercially.

A preferred affinity label is a non-variable portion of the immunoglobulin molecule. Typically, such portions comprise at least a functionally operative CH2 and CH3 domain of the constant region of an immunoglobulin heavy chain. Fusions are also made using the carboxyl terminus of the Fc portion of a constant domain, or a region immediately amino-terminal to the CH1 of the heavy or light chain. Suitable immunoglobulin-based affinity label may be obtained from IgG-1, -2, -3, or -4 subtypes, IgA, IgE, IgD, or IgM, but preferably IgG1. Preferably, a human immunoglobulin is used when the HPBF is intended

for *in vivo* use for humans. Many DNA encoding immunoglobulin light or heavy chain constant regions is known or readily available from cDNA libraries. See, for example, Adams *et al.*, Biochemistry, 1980, 19:2711-2719; Gough *et al.*, 1980, Biochemistry, 19:2702-2710; Dolby *et al.*, 1980, Proc. Natl. Acad. Sci. U.S.A., 77:6027-6031; Rice *et al.*, 1982, Proc. Natl. Acad. Sci. U.S.A., 79:7862-7865; Falkner *et al.*, 1982, Nature, 298:286-288; and Morrison *et al.*, 1984, Ann. Rev. Immunol, 2:239-256. Because many immunological reagents and labeling systems are available for the detection of immunoglobulins, the HPBF–Ig fusion protein can readily be detected and quantified by a variety of immunological techniques known in the art, such as the use of enzyme-linked immunosorbent assay (ELISA), immunoprecipitation, fluorescence activated cell sorting (FACS), etc. Similarly, if the affinity label is an epitope with readily available antibodies, such reagents can be used with the techniques mentioned above to detect, quantitate, and isolate the HPBF containing the affinity label. In many instances, there is no need to develop specific antibodies to the HPBF.

A particularly preferred embodiment is a fusion of an HSP peptide-binding fragment to the hinge, the CH2 and CH3 domains of human immunoglobulin G-1 (IgG-1; see Bowen *et al.*,1996, J. Immunol. 156:442-49). This hinge region contains three cysteine residues which are normally involved in disulfide bonding with other cysteines in the Ig molecule. Since none of the cysteines are required for the peptide to function as a tag, one or more of these cysteine residues may optionally be substituted by another amino acid residue, such as for example, serine.

Various leader sequences known in the art can be used for the efficient secretion of HPBF from bacterial and mammalian cells (von Heijne, 1985, J. Mol. Biol. 184:99-105). Leader peptides are selected based on the intended host cell, and may include bacterial, yeast, viral, animal, and mammalian sequences. For example, the herpes virus glycoprotein D leader peptide is suitable for use in a variety of mammalian cells. A preferred leader peptide for use in mammalian cells can be obtained from the V-J2-C region of the mouse immunoglobulin kappa chain (Bernard *et al.*, 1981, Proc. Natl. Acad. Sci. 78:5812-5816). Preferred leader sequences for targeting HPBF expression in bacterial cells include, but are not limited to, the leader sequences of the *E.coli* proteins OmpA (Hobom *et al.*, 1995, Dev. Biol. Stand. 84:255-262), Pho A (Oka *et al.*, 1985, Proc. Natl. Acad. Sci 82:7212-16), OmpT (Johnson *et al.*, 1996, Protein Expression 7:104-113), LamB and OmpF (Hoffman & Wright, 1985, Proc. Natl. Acad. Sci. USA 82:5107-5111), β-lactamase (Kadonaga *et al.*, 1984, J. Biol. Chem. 259:2149-54), enterotoxins (Morioka-Fujimoto *et al.*, 1991, J. Biol. Chem. 266:1728-32), and the *Staphylococcus aureus* protein A (Abrahmsen *et al.*, 1986, Nucleic Acids Res. 14:7487-7500), and the *B. subtilis* endoglucanase (Lo *et al.*, Appl. Environ. Microbiol. 54:2287-2292), as well as artificial and synthetic signal

sequences (MacIntyre *et al.*, 1990, Mol. Gen. Genet. 221:466-74; Kaiser *et al.*, 1987, Science, 235:312-317).

DNA sequences encoding a desired affinity label or leader peptide, which may be readily obtained from libraries, produced synthetically, or may be available from commercial suppliers, are suitable for the practice of this invention. Such methods are well known in the art.

5.2.1.2 Recombinant Expression

In various embodiments of the invention, sequences encoding an HPBF are inserted into an expression vector for propagation and expression in recombinant cells.

An expression construct, as used herein, refers to a nucleotide sequence encoding an HPBF operably associated with one or more regulatory regions which allows expression of the HPBF in an appropriate host cell. "Operably-associated" refers to an association in which the regulatory regions and the HPBF sequence to be expressed are joined and positioned in such a way as to permit transcription, and ultimately, translation.

Vectors based on *E. coli* are the most popular and versatile systems for high level expression of foreign proteins (Makrides, 1996, Microbiol Rev, 60:512-538). Non-limiting examples of regulatory regions that can be used for expression in *E. coli* may include but not limited to *lac, trp, lpp, phoA, recA, tac,* λP_L and phage T3 and T7 promoters (Makrides, 1996, Microbiol Rev, 60:512-538). Non-limiting examples of prokaryotic expression vectors may include the λgt vector series such as λgt11 (Huynh *et al.*, 1984 in "DNA Cloning Techniques", Vol. I: A Practical Approach (D. Glover, ed.), pp. 49-78, IRL Press, Oxford), and the pET vector series (Studier *et al.*, 1990, Methods Enzymol., 185:60-89). However, a potential drawback of a prokaryotic host-vector system is the inability to perform many of the post-translational processing events of mammalian cells. Thus, an eukaryotic host-vector system is preferred, a mammalian host-vector system is more preferred, and a human host-vector system is the most preferred.

The regulatory regions necessary for transcription of the HPBF can be provided by the expression vector. A translation initiation codon (ATG) may also be provided to express a nucleotide sequence encoding an HPBF that lacks an initiation codon. In a compatible host-construct system, cellular proteins required for transcription, such as RNA polymerase and transcription factors, will bind to the regulatory regions on the expression construct to effect transcription of the HPBF sequence in the host organism. The precise nature of the regulatory regions needed for gene expression may vary from host cell to host cell. Generally, a promoter is required which is capable of binding RNA polymerase to initiate the transcription of an operably-associated nucleic acid sequence. Such regulatory regions may include those 5'-non-coding sequences involved with initiation of transcription

and translation, such as the TATA box, the cap site, a CAAT box, and the like. The non-coding region 3' to the coding sequence may contain transcriptional termination regulatory sequences, such as terminators and polyadenylation sites.

Both constitutive and inducible regulatory regions may be used for expression of the HPBF. It may be desirable to use inducible promoters when the conditions optimal for growth of the recombinant cells and the conditions for high level expression of the HPBF are different. Examples of useful regulatory regions are provided in the next section below.

For expression of HSP peptide-binding fragments in mammalian host cells, a variety of regulatory regions can be used, for example, the SV40 early and late promoters, the cytomegalovirus (CMV) immediate early promoter, and the Rous sarcoma virus long terminal repeat (RSV-LTR) promoter. Inducible promoters that may be useful in mammalian cells include but are not limited to those associated with the metallothionein II gene, mouse mammary tumor virus glucocorticoid responsive long terminal repeats (MMTV-LTR), the β-interferon gene, and the Hsp70 gene (Williams *et al.*, 1989, Cancer Res. 49:2735-42 ; Taylor *et al.*, 1990, Mol. Cell Biol., 10:165-75). It may be advantageous to use heat shock promoters or stress promoters to drive expression of the HPBF in recombinant host cells.

The following animal regulatory regions, which exhibit tissue specificity and have been utilized in transgenic animals, can also be used in tumor cells of a particular tissue type: elastase I gene control region which is active in pancreatic acinar cells (Swift *et al.*, 1984, Cell 38:639-646; Ornitz *et al.*, 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl *et al.*, 1984, Cell 38:647-658; Adames *et al.*, 1985, Nature 318:533-538; Alexander *et al.*, 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder *et al.*, 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert *et al.*, 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf *et al.*, 1985, Mol. Cell. Biol. 5:1639-1648; Hammer *et al.*, 1987, Science 235:53-58; alpha 1-antitrypsin gene control region which is active in the liver (Kelsey *et al.*, 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram *et al.*, 1985, Nature 315:338-340; Kollias *et al.*, 1986, Cell 46:89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead *et al.*, 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985,

Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason *et al.*, 1986, Science 234:1372-1378).

The efficiency of expression of the HPBF in a host cell may be enhanced by the inclusion of appropriate transcription enhancer elements in the expression vector, such as those found in SV40 virus, Hepatitis B virus, cytomegalovirus, immunoglobulin genes, metallothionein, β-actin (see Bittner *et al.*, 1987, Methods in Enzymol. 153:516-544; Gorman, 1990, Curr. Op. in Biotechnol. 1:36-47).

The expression vector may also contain sequences that permit maintenance and replication of the vector in more than one type of host cell, or integration of the vector into the host chromosome. Such sequences may include but are not limited to replication origins, autonomously replicating sequences (ARS), centromere DNA, and telomere DNA. It may also be advantageous to use shuttle vectors that can be replicated and maintained in at least two types of host cells.

In addition, the expression vector may contain selectable or screenable marker genes for initially isolating or identifying host cells that contain DNA encoding an HPBF. For long term, high yield production of HPBF–peptide complexes, stable expression in mammalian cells is preferred. A number of selection systems may be used for mammalian cells, including, but not limited, to the Herpes simplex virus thymidine kinase (Wigler *et al.*, 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalski and Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy *et al.*, 1980, Cell 22:817) genes can be employed in *tk*$^-$, *hgprt*$^-$ or *aprt*$^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dihydrofolate reductase (*dhfr*), which confers resistance to methotrexate (Wigler *et al.*, 1980, Natl. Acad. Sci. USA 77:3567; O'Hare *et al.*, 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neomycin phosphotransferase (neo), which confers resistance to the aminoglycoside G-418 (Colberre-Garapin *et al.*, 1981, J. Mol. Biol. 150:1); and hygromycin phosphotransferase (hyg), which confers resistance to hygromycin (Santerre *et al.*, 1984, Gene 30:147). Other selectable markers, such as but not limited to histidinol and Zeocin™ can also be used.

In order to insert the HPBF DNA sequence into the cloning site of a vector, DNA sequences with regulatory functions, such as promoters, must be attached to DNA sequences encoding the HSP peptide-binding region. To do this, linkers or adapters providing the appropriate compatible restriction sites may be ligated to the ends of cDNA or synthetic DNA encoding an HPBF, by techniques well known in the art (Wu *et al.*, 1987, Methods in Enzymol 152:343-349). Cleavage with a restriction enzyme can be followed by modification to create blunt ends by digesting back or filling in single-stranded DNA

termini before ligation. Alternatively, a desired restriction enzyme site can be introduced into a fragment of DNA by amplification of the DNA by use of PCR with primers containing the desired restriction enzyme site.

An expression construct comprising an HPBF sequence operably associated with regulatory regions can be directly introduced into appropriate host cells for expression and production of HPBF-peptide complexes without further cloning (see, for example, U.S. Patent No. 5,580,859). The expression constructs may also contain DNA sequences that facilitate integration of the HPBF sequence into the genome of the host cell, *e.g.*, via homologous recombination. In this instance, it is not necessary to employ an expression vector comprising a replication origin suitable for appropriate host cells in order to propagate and express the HPBF in the host cells.

Expression constructs containing cloned nucleotide sequence encoding HSP peptide–binding fragments can be introduced into the host cell by a variety of techniques known in the art, including but not limited to, for prokaryotic cells, bacterial transformation (Hanahan, 1985, in DNA Cloning, A Practical Approach, 1:109-136), and for eukaryotic cells, calcium phosphate mediated transfection (Wigler *et al.*, 1977, Cell 11:223-232), liposome-mediated transfection (Schaefer-Ridder *et al.*, 1982, Science 215:166-168), electroporation (Wolff *et al.*, 1987, Proc Natl Acad Sci 84:3344), and microinjection (Cappechi, 1980, Cell 22:479-488). Co-expression of an HPBF and an antigen in the same host cell can be achieved by essentially the same methods.

For long term, high yield production of properly processed HPBFs or HPBF–peptide complexes, stable expression in mammalian cells is preferred. Cell lines that stably express HPBFs or HPBF–peptide complexes may be engineered by using a vector that contains a selectable marker. By way of example but not limitation, following the introduction of the expression constructs, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the expression construct confers resistance to the selection and optimally allows cells to stably integrate the expression construct into their chromosomes and to grow in culture and to be expanded into cell lines. Such cells can be cultured for a long period of time while HPBF is expressed continuously.

The recombinant cells may be cultured under standard conditions of temperature, incubation time, optical density, and media composition. Alternatively, a recombinant antigenic cells may be cultured under conditions emulating the nutritional and physiological requirements of the cancer cell or infected cell. However, conditions for growth of recombinant cells may be different from those for expression of HPBFs and antigenic proteins. Modified culture conditions and media may also be used to enhance production of HSP–peptide complexes. For example, recombinant cells containing HPBFs

with their cognate promoters may be exposed to heat or other environmental stress, or chemical stress. Any techniques known in the art may be applied to establish the optimal conditions for producing HPBF or HPBF–peptide complexes.

5.2.1.3 Purification Methods for Recombinant HSP Peptide-Binding Fragments

Generally, the HSP peptide-binding fragments of the invention can be recovered and purified from recombinant cell cultures by known methods, including ammonium sulfate precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, immunoaffinity chromatography, hydroxyapatite chromatography, and lectin chromatography.

The invention provides methods for purification of recombinant HPBFs by affinity purification, based on the properties of the affinity label present on the HSP peptide-binding fragment. One approach is based on specific molecular interactions between a tag and its binding partner. The other approach relies on the immunospecific binding of an antibody to an epitope present on the tag. The principle of affinity chromatography well known in the art is generally applicable to both of these approaches.

Described below are several methods based on specific molecular interactions of a tag and its binding partner.

A method that is generally applicable to purifying recombinant HSPs that are fused to the constant regions of immunoglobulin is protein A affinity chromatography, a technique that is well known in the art. Staphylococcus protein A is a 42 kD polypeptide that binds specifically to a region located between the second and third constant regions of heavy chain immunoglobulins. Because of the Fc domains of different classes, subclasses and species of immunoglobulins, affinity of protein A for human Fc regions is strong, but may vary with other species. Subclasses that are less preferred include human IgG-3, and most rat subclasses. For certain subclasses, protein G (of Streptococci) may be used in place of protein A in the purification. Protein-A sepharose (Pharmacia or Biorad) is a commonly used solid phase for affinity purification of antibodies, and can be used essentially in the same manner for the purification of HPBF fused to an immunoglobulin Fc fragment. Secreted HPBF present in cell supernatant binds specifically to protein A on the solid phase, while the contaminants are washed away. Bound HPBF can be eluted by various buffer systems known in the art, including a succession of citrate, acetate and glycine-HCl buffers which gradually lowers the pH. This method is less preferred if the recombinant cells also produce antibodies which will be copurified with the HPBF. See, for example, Langone, 1982, J. Immunol. meth. 51:3; Wilchek *et al.*, 1982, Biochem. Intl. 4:629; Sjobring *et al.*,

1991, J. Biol. Chem. 26:399; page 617-618, in Antibodies A Laboratory Manual, edited by Harlow and Lane, Cold Spring Harbor laboratory, 1988.

Alternatively, a polyhistidine tag may be used, in which case, the HSP peptide-binding fragment can be purified by metal chelate chromatography. The polyhistidine tag, usually a sequence of six histidines, has a high affinity for divalent metal ions, such as nickel ions (Ni^{2+}), which can be immobilized on a solid phase, such as nitrilotriacetic acid matrices. Polyhistidine has a well characterized affinity for Ni^{2+}-NTA-agarose, and can be eluted with either of two mild treatments: imidazole (0.1-0.2 M) will effectively compete with the resin for binding sites; or lowering the pH just below 6.0 will protonate the histidine side-chains and disrupt the binding. The purification method comprises loading the cell culture supernatant onto the Ni^{2+}-NTA-agarose column, washing the contaminants through, and eluting the HPBF with imidazole or weak acid. Ni^{2+}-NTA-agarose can be obtained from commercial suppliers such as Sigma (St. Louis) and Qiagen. Antibodies that recognize the polyhistidine tag are also available which can be used to detect and quantify the HPBF.

Another exemplary affinity label that can be used is the glutathione-S-transferase (GST) sequence, originally cloned from the helminth, *Schistosoma japonicum*. In general, an HSP peptide-binding fragment–GST fusion expressed in a prokaryotic host cell, such as *E. coli*, can be purified from the cell culture supernatant by absorption with glutathione agarose beads, followed by elution in the presence of free reduced glutathione at neutral pH. Denaturing conditions are not required at any stage during purification, and therefore, it may be desirable for use in the loading of immobilized HSP peptide-binding fragments with antigenic peptides. Moreover, since GST is known to form dimers under certain conditions, dimeric HPBFs may be obtained. See, Smith, 1993, Methods Mol. Cell Bio. 4:220-229.

Another useful affinity label that can be used is the maltose binding protein (MBP) of *E. coli*, which is encoded by the *malE* gene. The secreted HPBF-MBP present in the cell supernatant binds to amylose resin while contaminants are washed away. The bound HPBF-MBP is eluted from the amylose resin by maltose. See, for example, Guan *et al.*, 1987, Gene 67:21-30.

The second approach for purifying HSP peptide-binding fragments is applicable to affinity labels that contain an epitope for which polyclonal or monoclonal antibodies are available. Various methods known in the art for purification of protein by immunospecific binding, such as immunoaffinity chromatography, and immunoprecipitation, can be used. See, for example, Chapter 13 in "Antibodies A Laboratory Manual", 1988, Harlow and Lane, (eds.), Cold Spring Harbor Laboratory, N.Y.

and Chapter 8, Sections I and II, in "Current Protocols in Immunology", 1991, Coligan *et al.* (eds.), John Wiley,; the disclosure of which are both incorporated by reference herein.

The embodiments described above may be used to recover and purify HPBF–peptide complexes from the cell culture medium of mammalian cells, such as human cells expressing an HSP peptide-binding fragment of the invention. The methods can be adapted to perform medium and large scale purification of an HSP peptide-binding fragment and/or fragment-peptide complexes. Methods that do not require lowering pH or denaturing conditions are most preferred for purification of HPBF-peptide complexes. The methods may be used to isolate HSP peptide-binding fragments from eukaryotic cells, for example, cancer cells, tissues, isolated cells, or immortalized eukaryote cell lines infected with an intracellular pathogen, or cells obtained from a subject infected with a pathogen.

5.2.1.4 Host–Vector Systems

Described herein are systems of vectors and host cells that can be used for the expression of HSP peptide-binding fragments. A variety of expression vectors may be used in the present invention which include, but are not limited to, plasmids, cosmids, phage, phagemids, or modified viruses. Typically, such expression vectors comprise a functional origin of replication for propagation of the vector in an appropriate host cell, one or more restriction endonuclease sites for insertion of the HPBF gene sequence, and one or more selection markers. The expression vector must be used with a compatible host cell which may be derived from a prokaryotic or an eukaryotic organism including but not limited to bacteria, yeasts, insects, mammals, and humans.

Expression constructs and vectors are introduced into host cells for the purpose of producing an HSP peptide-binding fragment. Any cell type that can produce HSPs and is compatible with the expression vector may be used, including those that have been cultured *in vitro* or genetically engineered. Host cells may be obtained from normal or affected subjects, including healthy humans, cancer patients, and patients with an infectious disease, private laboratory deposits, public culture collections such as the American Type Culture Collection, or from commercial suppliers.

Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. A host cell may be chosen which modifies and processes the expressed gene products in a specific fashion similar to the way the recipient processes HSPs. For the purpose of producing large amounts of HSP, it is preferable that the type of host cell used in the present invention has been used for expression of heterologous genes, and is reasonably well characterized and developed for large-scale production processes. In a specific embodiment, the host cells are from the same

patient to whom HPBF–peptide complexes or recombinant cells expressing HPBF–peptide complexes are going to be administered. Otherwise said, the cells used to express the HPBF and used subsequently to administer immunotherapy to a subject are autologous to the subject.

Preferred mammalian host cells include but are not limited to those derived from humans, monkeys and rodents, (see, for example, Kriegler M. in "Gene Transfer and Expression: A Laboratory Manual", New York, Freeman & Co. 1990), such as monkey kidney cell line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293, 293-EBNA, or 293 cells subcloned for growth in suspension culture, Graham *et al.*, J. Gen. Virol., 36:59, 1977; baby hamster kidney cells (BHK, ATCC CCL 10); chinese hamster ovary-cells-DHFR (CHO, Urlaub and Chasin. Proc. Natl. Acad. Sci. 77; 4216, 1980); mouse sertoli cells (Mather, Biol. Reprod. 23:243-251, 1980); mouse fibroblast cells (NIH-3T3), monkey kidney cells (CVI ATCC CCL 70); african green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); and mouse mammary tumor cells (MMT 060562, ATCC CCL51). Exemplary cancer cell types used for demonstrating the utility of recombinant cells (producing HPBF-peptide complexes) as a cancer vaccine are provided as follows: mouse fibroblast cell line, NIH3T3, mouse Lewis lung carcinoma cell line, LLC, mouse mastocytoma cell line, P815, mouse lymphoma cell line, EL4 and its ovalbumin transfectant, E.G7, mouse melanoma cell line, B16F10, mouse fibrosarcoma cell line, MC57, and human small cell lung carcinoma cell lines, SCLC#2 and SCLC#7.

A number of viral-based expression systems may also be utilized with mammalian cells to produce HSP peptide-binding fragments. Vectors using DNA virus backbones have been derived from simian virus 40 (SV40) (Hamer *et al.*, 1979, Cell 17:725), adenovirus (Van Doren *et al.*, 1984, Mol Cell Biol 4:1653), adeno-associated virus (McLaughlin *et al.*, 1988, J Virol 62:1963), and bovine papillomas virus (Zinn *et al.*, 1982, Proc Natl Acad Sci 79:4897). In cases where an adenovirus is used as an expression vector, the donor DNA sequence may be ligated to an adenovirus transcription/translation control region, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing heterologous products in infected hosts (see *e.g.*, Logan and Shenk, 1984, Proc. Natl. Acad. Sci. (USA) 81:3655-3659).

Bovine papillomavirus (BPV) can infect many higher vertebrates, including man, and its DNA replicates as an episome. A number of shuttle vectors have been

developed for recombinant gene expression which exist as stable, multicopy (20-300 copies/cell) extrachromosomal elements in mammalian cells. Typically, these vectors contain a segment of BPV DNA (the entire genome or a 69% transforming fragment), a promoter with a broad host range, a polyadenylation signal, splice signals, a selectable marker, and "poisonless" plasmid sequences that allow the vector to be propagated in *E. coli*. Following construction and amplification in bacteria, the expression gene construct is transfected into cultured mammalian cells, for example, by the techniques of calcium phosphate coprecipitation or electroporation. For those host cells that do not manifest a transformed phenotype, selection of transformants is achieved by use of a dominant selectable marker, such as histidinol and G418 resistance. For example, BPV vectors such as pBCMGSNeo and pBCMGHis may be used to express HPBF sequences (Karasuyama *et al.*, Eur. J. Immunol. 18:97-104; Ohe *et al.*, Human Gene Therapy, 6:325-33) which may then be transfected into a diverse range of cell types for expression of the HPBF.

Alternatively, the vaccinia 7.5K promoter may be used (see, *e.g.*, Mackett *et al.*, 1982, Proc. Natl. Acad. Sci. (USA) 79:7415-7419; Mackett *et al.*, 1984, J. Virol. 49:857-864; Panicali *et al.*, 1982, Proc. Natl. Acad. Sci. 79:4927-4931.) In cases where a human host cell is used, vectors based on the Epstein-Barr virus (EBV) origin (OriP) and EBV nuclear antigen 1 (EBNA-1; a trans-acting replication factor) may be used. Such vectors can be used with a broad range of human host cells, *e.g.*, EBO-pCD (Spickofsky *et al.*, 1990, DNA Prot Eng Tech 2:14-18), pDR2 and λDR2 (available from Clontech Laboratories).

HPBFs may also be made with a retrovirus-based expression system. In contrast to transfection, retroviruses can efficiently infect and transfer genes to a wide range of cell types including, for example, primary hematopoietic cells. In retroviruses such as Moloney murine leukemia virus, most of the viral gene sequences can be removed and replaced with nucleic acid sequences encoding the HSP peptide-binding domain, while the missing viral functions can be supplied in *trans*. The host range for infection by a retroviral vector can also be manipulated by the choice of envelope used for vector packaging.

For example, a retroviral vector can comprise a 5' long terminal repeat (LTR), a 3' LTR, a packaging signal, a bacterial origin of replication, and a selectable marker. The HPBF DNA is inserted into a position between the 5' LTR and 3' LTR, such that transcription from the 5' LTR promoter transcribes the cloned DNA. The 5' LTR comprises a promoter, including but not limited to an LTR promoter, an R region, a U5 region and a primer binding site, in that order. Nucleotide sequences of these LTR elements are well known in the art. A heterologous promoter as well as multiple drug selection markers may also be included in the expression vector to facilitate selection of infected cells (see McLauchlin *et al.*, 1990, Prog. Nucleic Acid Res. and Molec. Biol. 38:91-135;

Morgenstern *et al.*, 1990, Nucleic Acid Res. 18:3587-3596; Choulika *et al.*, 1996, J. Virol 70:1792-1798; Boesen *et al.*, 1994, Biotherapy 6:291-302; Salmons and Gunzberg, 1993, Human Gene Therapy 4:129-141; and Grossman and Wilson, 1993, Curr. Opin. in Genetics and Devel. 3:110-114).

Other useful eukaryotic host-vector system may include yeast and insect systems. In yeast, a number of vectors containing constitutive or inducible promoters may be used with *Saccharomyces cerevisiae* (baker's yeast), *Schizosaccharomyces pombe* (fission yeast), *Pichia pastoris,* and *Hansenula polymorpha* (methylotropic yeasts). For a review see, "Current Protocols in Molecular Biology", Vol. 2, 1988, Ausubel *et al.* (eds.), Greene Publish. Assoc. & Wiley Interscience, Ch. 13; Grant *et al.*, 1987, Expression and Secretion Vectors for Yeast, 1987, in "Methods in Enzymology", Wu and Grossman (eds.), Acad. Press, N.Y., Vol. 153, pp. 516-544; Glover, 1986, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3; and Bitter, 1987, Heterologous Gene Expression in Yeast, in "Methods in Enzymology", Berger and Kimmel (eds.), Acad. Press, N.Y., Vol. 152, pp. 673-684; and "The Molecular Biology of the Yeast *Saccharomyces*", 1982, Strathern *et al.* (eds.), Cold Spring Harbor Press, Vols. I and II.

In an insect system a baculovirus, *Autographa californica* nuclear polyhidrosis virus (AcNPV), can be used as a vector to express an HPBF in *Spodoptera frugiperda* cells. The HPBF DNA may be cloned into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). These recombinant viruses are then used to infect host cells in which the inserted DNA is expressed (see, *e.g.*, Smith *et al.*, 1983, J. Virol. 46:584; Smith, U.S. Patent No. 4,215,051).

Any of the cloning and expression vectors described herein may be synthesized and assembled from known DNA sequences by techniques well known in the art. The regulatory regions and enhancer elements can be of a variety of origins, both natural and synthetic. Some vectors and host cells may be obtained commercially. Non-limiting examples of useful vectors are described in Appendix 5 of Current Protocols in Molecular Biology, 1988, ed. Ausubel *et al.*, Greene Publish. Assoc. & Wiley Interscience, which is incorporated herein by reference; and the catalogs of commercial suppliers such as Clontech Laboratories, Stratagene Inc., and Invitrogen, Inc.

5.2.2 Synthetic Production of HSP Peptide-Binding Fragments

An alternative to producing HPBF by recombinant techniques is peptide synthesis. For example, a peptide corresponding to a portion of an HSP comprising the substrate-binding domain, or which binds peptides *in vitro*, can be synthesized by use of a

peptide synthesizer. Conventional peptide synthesis may be used or other synthetic protocols well known in the art.

In addition, analogs and derivatives of HPBF protein can be chemically synthesized. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the HSP sequence. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, α-amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, γ-Abu, ϵ-Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β-methyl amino acids, Cα-methyl amino acids, Nα-methyl amino acids, and amino acid analogs in general.

Peptides having the amino acid sequence of a peptide-binding HPBF, or a mutant or derivative thereof, may be synthesized by solid-phase peptide synthesis using procedures similar to those described by Merrifield, 1963, J. Am. Chem. Soc., 85:2149. During synthesis, N-α-protected amino acids having protected side chains are added stepwise to a growing polypeptide chain linked by its C-terminal and to an insoluble polymeric support i.e., polystyrene beads. The peptides are synthesized by linking an amino group of an N-α-deprotected amino acid to an α-carboxyl group of an N-α-protected amino acid that has been activated by reacting it with a reagent such as dicyclohexylcarbodiimide. The attachment of a free amino group to the activated carboxyl leads to peptide bond formation. The most commonly used N-α-protecting groups include Boc which is acid labile and Fmoc which is base labile. Details of appropriate chemistries, resins, protecting groups, protected amino acids and reagents are well known in the art and so are not discussed in detail herein (*See*, Atherton, *et al.*, 1989, Solid Phase Peptide Synthesis: A Practical Approach, IRL Press, and Bodanszky, 1993, Peptide Chemistry, A Practical Textbook, 2nd Ed., Springer-Verlag).

Purification of the resulting HSP peptide-binding fragment is accomplished using conventional procedures, such as preparative HPLC using gel permeation, partition and/or ion exchange chromatography. The choice of appropriate matrices and buffers are well known in the art and so are not described in detail herein.

5.2.3 Production of HSP Peptide-binding Fragments by Proteolytic Cleavage

In an alternative embodiment, peptide-binding HSP fragments may be obtained by chemical or enzymatic cleavage of native or recombinant HSPs. Specific chemical cleavage can be performed by cyanogen bromide, $NaBH_4$, acetylation, formylation, oxidation, reduction, metabolic synthesis in the presence of tunicamycin, *etc.*.

Endoproteases that cleave at specific sites can also be used. Such proteases are known in the art, including, but not limited to, trypsin, α-chymotrypsin, V8 protease, papain, and proteinase K (see Ausubel *et al.*, (eds.), in "Current Protocols in Molecular Biology", Greene Publishing Associates and Wiley Interscience, New York, 17.4.6-17.4.8). The amino acid sequence of an HSP of interest can be examined for the recognition sites of these proteases. An enzyme is chosen which can release a peptide-binding domain or peptide-binding fragment. The HSP molecule is then incubated with the protease, under conditions that allow digestion by the protease and release of the specifically designated peptide-binding fragments. Alternatively, such protease digestions can be carried out blindly, i.e., not knowing which digestion product will contain the peptide-binding domain, using specific or general specificity proteases, such as proteinase K or pronase. The digestion products may be purified as described above, and subsequently tested for the ability to bind peptide or for immunogenicity. Methods for determining the immunogenicity of HSP complexes by cytotoxicity tests are described in Section 5.4.1.

5.3 HSP Peptide-binding Fragments – Antigenic Molecule Complexes

5.3.1 Preparation of Intracellular Complexes of HSP Peptide-Binding Fragments with Antigenic Molecules

Described herein are methods for purifying HSP peptide-binding fragments or HPBF–peptide complexes of the invention from recombinant cells, and, with minor modifications known in the art, the HSP peptide-binding fragment or fragment–peptide complexes from the cell culture. Recombinant cells include, for example, cells expressing antigenic molecules and recombinantly expressing heat shock protein or an HPBF. Such cells may be derived from a variety of sources, including, but not limited to, cells infected with an infectious agent and cancer cells.

The purification of HSP–peptide complexes from cell lysates has been described previously, see, for example, Udono *et al.*, 1993, J. Exp. Med. 178:1391-1396. The purification of Hsp90–peptide complexes and gp96–peptide complexes from cell lysates have been described, for example, in WO 95/24923, dated September 21, 1995, and WO 97/10000, dated March 20, 1997, which are incorporated herein by reference in their entirety.

The invention provides methods for purification of recombinant HPBF–peptide complexes by affinity purification, based on the properties of the affinity label present on the HPBF. One approach is based on specific molecular interactions between a tag and its binding partner. The other approach relies on the immunospecific binding of an antibody to an epitope present on the tag. The principle of affinity chromatography well known in the art is generally applicable to both of these approaches.

To produce HPBF–antigenic peptide complexes, a nucleotide sequence encoding an HPBF can be introduced into a cell. When an antigenic molecule is present in the cell, the HPBF can associate intracellularly with the antigenic molecule, forming a non-covalent complex of HPBF and the antigenic molecule. Cells into which an HPBF-encoding nucleotide sequence can be introduced, include, but are not limited to, epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes; blood cells such as T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells, *e.g.*, as obtained from bone marrow, umbilical cord blood, peripheral blood, fetal liver, etc. The choice of cell type depends on the type of tumor or infectious disease being treated or prevented, and can be determined by one of skill in the art.

In a specific embodiment, an expression construct comprising a nucleic acid sequence encoding the HSP peptide-binding fragment is introduced into an antigenic cell. As used herein, antigenic cells may include cells that are infected with an infectious agent or pathogen, cells infected with non-infectious or non-pathogenic forms of an infectious agent or pathogen (*e.g.*, by use of a helper infectious agent), cells infected by or engineered to express an attenuated form of an infectious agent or a non-pathogenic or replication-deficient variant of a pathogen, pre-neoplastic cells that are infected with a cancer-causing infectious agent, such as a virus, but which are not yet neoplastic; or antigenic cells that have been exposed to a mutagen or cancer-causing agent, such as, for example DNA-damaging agents, radiation, etc. Other cells that can be used are pre-neoplastic cells which are in transition from a normal to a neoplastic form as characterized by morphology, physiological or biochemical functions. Preferably, the cancer cells and pre-neoplastic cells used in the methods of the invention are of mammalian origin. Mammals contemplated by this aspect of the invention include humans, companion animals (*e.g.*, dogs and cats), livestock animals (*e.g.*, sheep, cattle, goats, pigs and horses), laboratory animals (*e.g.*, mice, rats and rabbits), and captive or free wild animals.

In various embodiments, any cancer cell, preferably a human cancer cell, can be used in the present methods for producing HPBF–peptide complexes. The cancer cells provide the antigenic peptides which become associated noncovalently with the expressed HPBF. HPBF-antigenic peptide complexes are then purified from the cells and used to treat such cancers. Cancers which can be treated or prevented with immunogenic compositions prepared by methods of the invention include, but are not limited to, tumors such as sarcomas and carcinomas. Examples of cancers that are amenable to the methods of the invention are listed in Section 5.4.2. Accordingly, any tissues or cells isolated from a pre-neoplastic lesion, a cancer, including cancer that has metastasized to multiple remote sites,

can be used in the present method. For example, cells found in abnormally growing tissue, circulating leukemic cells, metastatic lesions as well as solid tumor tissue can be used.

In another embodiment, cell lines derived from a pre-neoplastic lesion, cancer tissues or cancer cells can also be used, provided that the cells of the cell line have at least one or more antigenic determinants in common with antigens on the target cancer cells. Cancer tissues, cancer cells, cells infected with a cancer-causing agent, other pre-neoplastic cells, and cell lines of human origin are preferred.

Cancer and pre-neoplastic cells can be identified by any method known in the art. For example, cancer cells can be identified by morphology, enzyme assays, proliferation assays, cytogenetic characterization, DNA mapping, DNA sequencing, the presence of cancer-causing virus, or a history of exposure to mutagen or cancer-causing agent, imaging, etc. Cancer cells may also be obtained by surgery, endoscopy, or other biopsy techniques. If some distinctive characteristics of the cancer cells are known, they can also be obtained or purified by any biochemical or immunological methods known in the art, such as but not limited to affinity chromatography, and fluorescence activated cell sorting (*e.g.*, with fluorescently tagged antibody against an antigen expressed by the cancer cells).

Cancer tissues, cancer cells or cell lines may be obtained from a single individual or pooled from several individuals. It is not essential that clonal, homogeneous, or purified population of cancer cells be used. It is also not necessary to use cells of the ultimate target *in vivo* (*e.g.*, cells from the tumor of the intended recipient), so long as at least one or more antigenic determinants on the target cancer cells is present on the cells used for expression of the HPBF. In addition, cells derived from distant metastases may be used to prepare an immunogenic composition against the primary cancer. A mixture of cells can be used provided that a substantial number of cells in the mixture are cancer cells and share at least one antigenic determinant with the target cancer cell. In a specific embodiment, the cancer cells to be used in expressing an HPBF are purified.

5.3.1.1 Preparation of Hsp70–Peptide Complexes

The purification of Hsp70–peptide complexes has been described previously, see, for example, Udono *et al.*, 1993, J. Exp. Med. 178:1391-1396. The following procedure may be used, presented by way of example but not limitation, to purify Hsp70 complexes. Initially, tumor cells are suspended in 3 volumes of 1X Lysis buffer consisting of 5mM sodium phosphate buffer, pH 7, 150mM NaCl, 2mM $CaCl_2$, 2mM $MgCl_2$ and 1mM phenyl methyl sulfonyl fluoride (PMSF). Then, the pellet is sonicated, on ice, until >99% cells are lysed as determined by microscopic examination. As an alternative to sonication, the cells may be lysed by mechanical shearing and in this approach the cells typically are

resuspended in 30mM sodium bicarbonate pH 7.5, 1mM PMSF, incubated on ice for 20 minutes and then homogenized in a Dounce homogenizer until >95% cells are lysed.

Then the lysate is centrifuged at 1,000g for 10 minutes to remove unbroken cells, nuclei and other cellular debris. The resulting supernatant is recentrifuged at 100,000g for 90 minutes, the supernatant harvested and then mixed with Con A Sepharose equilibrated with phosphate buffered saline (PBS) containing 2mM Ca^{2+} and 2mM Mg^{2+}. When the cells are lysed by mechanical shearing the supernatant is diluted with an equal volume of 2X lysis buffer prior to mixing with Con A Sepharose. The supernatant is then allowed to bind to the Con A Sepharose for 2-3 hours at 4°C. The material that fails to bind is harvested and dialyzed for 36 hours (three times, 100 volumes each time) against 10mM Tris-Acetate pH 7.5, 0.1mM EDTA, 10mM NaCl, 1mM PMSF. Then the dialyzate is centrifuged at 17,000 rpm (Sorvall SS34 rotor) for 20 minutes. Then the resulting supernatant is harvested and applied to a Mono Q FPLC column equilibrated in 20mM Tris-acetate pH 7.5, 2mM NaCl, 0.1mM EDTA and 15mM 2-mercaptuethanol). The column is then developed with a 20mM to 500mM NaCl gradient and then eluted fractions fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and characterized by immunoblotting using an appropriate anti-Hsp70 antibody (such as from clone N27F3-4, from StressGen).

Fractions strongly immunoreactive with the anti-Hsp70 antibody are pooled and the Hsp70–peptide complexes precipitated with ammonium sulfate; specifically with a 50%-70% ammonium sulfate cut. The resulting precipitate is then harvested by centrifugation at 17,000 rpm (SS34 Sorvall rotor) and washed with 70% ammonium sulfate. The washed precipitate is then solubilized and any residual ammonium sulfate removed by gel filtration on a Sephadex^R G25 column (Pharmacia). If necessary the Hsp70 preparation thus obtained can be repurified through the Mono Q FPLC Column as described above.

The Hsp70–peptide complex can be purified to apparent homogeneity using this method. Typically 1 mg of Hsp70–peptide complex can be purified from 1 g of cells/tissue.

An improved method for purification of Hsp70–peptide complexes comprises contacting cellular proteins with ADP or a nonhydrolyzable analog of ATP affixed to a solid substrate, such that Hsp70 in the lysate can bind to the ADP or nonhydrolyzable ATP analog, and eluting the bound Hsp70. A preferred method uses column chromatography with ADP affixed to a solid substratum (*e.g.*, ADP-agarose). The resulting Hsp70 preparations are higher in purity and devoid of non-specifically bound peptides. The Hsp70 yields are also increased significantly by about more than 10 fold. Alternatively, chromatography with nonhydrolyzable analogs of ATP, instead of ADP, can be used for purification of Hsp70–peptide complexes. By way of example but not limitation,

purification of Hsp70–peptide complexes by ADP-agarose chromatography can be carried out as follows:

Meth A sarcoma cells (500 million cells) are homogenized in hypotonic buffer and the lysate is centrifuged at 100,000 g for 90 minutes at 4°C. The supernatant is applied to an ADP-agarose column. The column is washed in buffer and is eluted with 5 column volumes of 3 mM ADP. The Hsp70–peptide complexes elute in fractions 2 through 10 of the total 15 fractions which elute. The eluted fractions are analyzed by SDS-PAGE. The Hsp70–peptide complexes can be purified to apparent homogeneity using this procedure.

5.3.1.2 Preparation of Hsp90–Peptide Complexes

The purification of Hsp90–peptide complexes from cell lysates has been described, for example, in WO 95/24923, dated September 21, 1995, and WO 97/10000, dated March 20, 1997. A procedure that can be used, presented by way of example and not limitation, is as follows:

Initially, tumor cells are suspended in 3 volumes of 1X Lysis buffer consisting of 5mM sodium phosphate buffer (pH7), 150mM NaCl, 2mM $CaCl_2$, 2mM $MgCl_2$ and 1mM phenyl methyl sulfonyl fluoride (PMSF). Then, the pellet is sonicated, on ice, until >99% cells are lysed as determined by microscopic examination. As an alternative to sonication, the cells may be lysed by mechanical shearing and in this approach the cells typically are resuspended in 30mM sodium bicarbonate pH 7.5, 1mM PMSF, incubated on ice for 20 minutes and then homogenized in a Dounce homogenizer until >95% cells are lysed.

Then the lysate is centrifuged at 1,000g for 10 minutes to remove unbroken cells, nuclei and other cellular debris. The resulting supernatant is recentrifuged at 100,000g for 90 minutes, the supernatant harvested and then mixed with Con A Sepharose equilibrated with PBS containing 2mM Ca^{2+} and 2mM Mg^{2+}. When the cells are lysed by mechanical shearing the supernatant is diluted with an equal volume of 2X Lysis buffer prior to mixing with Con A Sepharose. The supernatant is then allowed to bind to the Con A Sepharose for 2-3 hours at 4°C. The material that fails to bind is harvested and dialyzed for 36 hours (three times, 100 volumes each time) against 20mM Sodium phosphate pH 7.4, 1mM EDTA, 250mM NaCl, 1mM PMSF. Then the dialyzate is centrifuged at 17,000 rpm (Sorvall SS34 rotor) for 20 minutes. Then the resulting supernatant is harvested and applied to a Mono Q FPLC column equilibrated with dialysis buffer. The proteins are then eluted with a salt gradient of 200mM to 600mM NaCl.

The eluted fractions are fractionated by SDS-PAGE and fractions containing the Hsp90–peptide complexes identified by immunoblotting using an anti-Hsp90 antibody

such as 3G3 (Affinity Bioreagents). Hsp90–peptide complexes can be purified to apparent homogeneity using this procedure. Typically, 150-200 μg of Hsp90–peptide complex can be purified from 1g of cells/tissue.

5.3.1.3 Preparation of gp96–Peptide Complexes

The purification of gp96–peptide complexes from cell lysates has been described, for example, in WO 95/24923, dated September 21, 1995, and WO 97/10000, dated March 20, 1997. A procedure that can be used, presented by way of example and not limitation, is as follows:

A cell pellet is resuspended in 3 volumes of buffer consisting of 30mM sodium bicarbonate buffer (pH 7.5) and 1mM PMSF and the cells allowed to swell on ice 20 minutes. The cell pellet is then homogenized in a Dounce homogenizer (the appropriate clearance of the homogenizer will vary according to each cell type) on ice until >95% cells are lysed.

The lysate is centrifuged at 1,000g for 10 minutes to remove unbroken cells, nuclei and other debris. The supernatant from this centrifugation step is then recentrifuged at 100,000g for 90 minutes. The gp96–peptide complex can be purified either from the 100,000 pellet or from the supernatant.

When purified from the supernatant, the supernatant is diluted with equal volume of 2X lysis buffer and the supernatant mixed for 2-3 hours at 4°C with ConA Sepharose equilibrated with PBS containing 2mM Ca^{2+} and 2mM Mg^{2+}. Then, the slurry is packed into a column and washed with 1X lysis buffer until the OD_{280} drops to baseline. Then, the column is washed with 1/3 column bed volume of 10% α-methyl mannoside (α-MM) dissolved in PBS containing 2mM Ca^{2+} and 2mM Mg^{2+}, the column sealed with a piece of parafilm, and incubated at 37°C for 15 minutes. Then the column is cooled to room temperature and the parafilm removed from the bottom of the column. Five column volumes of the α-MM buffer are applied to the column and the eluate analyzed by SDS-PAGE. Typically the resulting material is about 60-95% pure, however this depends upon the cell type and the tissue-to-lysis buffer ratio used. Then the sample is applied to a Mono Q FPLC column (Pharmacia) equilibrated with a buffer containing 20mM sodium phosphate, pH 7.4. The proteins are then eluted from the column with a 0-1M NaCl gradient and the gp96 fraction elutes between 400mM and 550mM NaCl.

The procedure, however, may be modified by two additional steps, used either alone or in combination, to consistently produce apparently homogeneous gp96-peptide complexes. One optional step involves an ammonium sulfate precipitation prior to the Con A purification step and the other optional step involves DEAE-Sepharose purification after the Con A purification step but before the Mono Q FPLC step.

In the first optional step, described by way of example as follows, the supernatant resulting from the 100,000g centrifugation step is brought to a final concentration of 50% ammonium sulfate by the addition of ammonium sulfate. The ammonium sulfate is added slowly while gently stirring the solution in a beaker placed in a tray of ice water. The solution is stirred from about 1/2 to 12 hours at 4°C and the resulting solution centrifuged at 6,000 rpm (Sorvall SS34 rotor). The supernatant resulting from this step is removed, brought to 70% ammonium sulfate saturation by the addition of ammonium sulfate solution, and centrifuged at 6,000 rpm (Sorvall SS34 rotor). The resulting pellet from this step is harvested and suspended in PBS containing 70% ammonium sulfate in order to rinse the pellet. This mixture is centrifuged at 6,000 rpm (Sorvall SS34 rotor) and the pellet dissolved in PBS containing 2mM Ca^{2+} and Mg^{2+}. Undissolved material is removed by a brief centrifugation at 15,000 rpm (Sorvall SS34 rotor). Then, the solution is mixed with Con A Sepharose and the procedure followed as before.

In the second optional step, described by way of example as follows, the gp96 containing fractions eluted from the Con A column are pooled and the buffer exchanged for 5mM sodium phosphate buffer, pH 7, 300mM NaCl by dialysis, or preferably by buffer exchange on a Sephadex G25 column. After buffer exchange, the solution is mixed with DEAE-Sepharose previously equilibrated with 5mM sodium phosphate buffer, pH 7, 300mM NaCl. The protein solution and the beads are mixed gently for 1 hour and poured into a column. Then, the column is washed with 5mM sodium phosphate buffer, pH 7, 300mM NaCl, until the absorbance at 280nm drops to baseline. Then, the bound protein is eluted from the column with five volumes of 5mM sodium phosphate buffer, pH 7, 700mM NaCl. Protein containing fractions are pooled and diluted with 5mM sodium phosphate buffer, pH 7 in order to lower the salt concentration to 175mM. The resulting material then is applied to the Mono Q FPLC column (Pharmacia) equilibrated with 5mM sodium phosphate buffer, pH 7 and the protein that binds to the Mono Q FPLC column (Pharmacia) is eluted as described before.

It is appreciated, however, that one skilled in the art may assess, by routine experimentation, the benefit of incorporating the second optional step into the purification protocol. In addition, it is appreciated also that the benefit of adding each of the optional steps will depend upon the source of the starting material.

When the gp96 fraction is isolated from the 100,000g pellet, the pellet is suspended in 5 volumes of PBS containing either 1% sodium deoxycholate or 1% octyl glucopyranoside (but without the Mg^{2+} and Ca^{2+}) and incubated on ice for 1 hour. The suspension is centrifuged at 20,000g for 30 minutes and the resulting supernatant dialyzed against several changes of PBS (also without the Mg^{2+} and Ca^{2+}) to remove the detergent. The dialysate is centrifuged at 100,000g for 90 minutes, the supernatant harvested, and

calcium and magnesium are added to the supernatant to give final concentrations of 2mM, respectively. Then the sample is purified by either the unmodified or the modified method for isolating gp96–peptide complex from the 100,000g supernatant, see above.

The gp96–peptide complexes can be purified to apparent homogeneity using this procedure. About 10-20μg of gp96 can be isolated from 1g cells/tissue.

5.3.2 *In Vitro* Complexing

In another embodiment, complexes of HPBFs to antigenic molecules are produced *in vitro*. Immunogenic HPBF–peptide complexes can be generated *in vitro* by noncovalent coupling of an HPBF with an antigenic peptide. Antigenic molecules may be isolated from various sources, chemically synthesized, or produced recombinantly. Procedures for forming such HSP–peptide complexes and methods for isolating antigenic peptides are described in detail herein. Such methods can be readily adapted for medium or large scale production of the immunotherapeutic or prophylactic vaccines of the invention.

A preferred, exemplary protocol for noncovalently complexing an HPBF and an antigenic molecule *in vitro* is provided herein.

It may be advantageous to use HPBFs that are reversibly bound to a solid phase to facilitate buffer exchange, washings and isolation of the complexes before or after the complexing reaction. Prior to complexing, the HPBFs may be pretreated with ATP or low pH to remove any peptides that may be associated with the HPBF of interest. When the ATP procedure is used, excess ATP is removed from the preparation by the addition of apyranase as described by Levy *et al.*, 1991, Cell 67:265-274. When the low pH procedure is used, the buffer is readjusted to neutral pH by the addition of pH modifying reagents.

The antigenic molecules (1μg) and the pretreated HPBF (9μg) are admixed to give an approximately 5:1 antigenic molecule : HPBF molar ratio. Then, the mixture is incubated for 15 minutes to 3 hours at 25°C to 45°C in a suitable binding buffer such as one containing 20mM sodium phosphate, pH 7.2, 350mM NaCl, 3mM $MgCl_2$ and 1mM phenyl methyl sulfonyl fluoride (PMSF). The preparations are centrifuged through a Centricon 10 assembly (Millipore) to remove any unbound peptide. If the HPBF is bound to a solid phase, the HPBF-peptide complexes formed can be washed free of unbounded peptide prior to eluting the HPBF-peptide complex off the solid phase. The association of the peptides with the HPBF can be assayed by SDS-PAGE. This is the preferred method for *in vitro* complexing of peptides isolated from MHC-peptide complexes of peptides disassociated from endogenous HSP-peptide complexes.

In an alternative embodiment, preferred for producing complexes of Hsp70 fragments to exogenous antigenic molecules such as proteins, 5-10 micrograms of purified HPBF is incubated with equimolar quantities of the antigenic molecule in 20mM sodium

phosphate buffer pH 7.5, 0.5M NaCl, 3mM $MgCl_2$ and 1mM ADP in a volume of 100 microliter at 37°C for 1 hr. This incubation mixture is further diluted to 1 ml in phosphate-buffered saline.

In another alternative embodiment of the invention, preferred for producing complexes of gp96 fragment to peptides, 5-10 micrograms of gp96 fragment immobilized by its affinity tag to a solid phase is incubated with equimolar or excess quantities of the antigenic peptide in a suitable buffer, such as one containing 20mM sodium phosphate buffer pH 7.5, 0.5M NaCl, 3nM MgCl2 at about 50°C for about 10 minutes. For example, modified gp96 containing the Ig tag can be immobilized to protein A-Sepharose for this procedure. This incubation mixture is then further incubated for about 30 minutes at room temperature. The solid phase with the bound HPBF-peptide complexes is washed several times to remove any unbound peptide. The HPBF-peptide complexes is then eluted from the solid phase by the appropriate technique.

Following complexing, the immunogenic HSP-antigenic molecule complexes can optionally be assayed *in vitro* using, for example, the mixed lymphocyte target cell assay (MLTC) described below. Once immunogenic complexes have been isolated they can be optionally characterized further in animal models using the preferred administration protocols and excipients discussed below.

5.3.3 Sources of Antigenic Molecules

Antigens, or antigenic portions thereof, specific to one or more types of cancer or infected cells, can be chosen from among those known in the art. Alternatively, such antigens can be selected for their antigenicity or their immunogenicity, as determined by immunoassays or by their ability to generate an immune response.

5.3.3.1 Exogenous Antigenic Molecules

Preferably, where it is desired to treat or prevent cancer, known tumor-specific antigens or fragments or derivatives thereof are used. For example, such tumor specific or tumor-associated antigens include but are not limited to KS 1/4 pan-carcinoma antigen (Perez and Walker, 1990, J. Immunol. 142:3662-3667; Bumal, 1988, Hybridoma 7(4):407-415); ovarian carcinoma antigen (CA125) (Yu, *et al.*, 1991, Cancer Res. 51(2):468-475); prostatic acid phosphate (Tailer, *et al.*, 1990, Nucl. Acids Res. 18(16):4928); prostate specific antigen (Henttu and Vihko, 1989, Biochem. Biophys. Res. Comm. 160(2):903-910; Israeli, *et al.*, 1993, Cancer Res. 53:227-230); melanoma-associated antigen p97 (Estin, *et al.*, 1989, J. Natl. Cancer Inst. 81(6):445-446); melanoma antigen gp75 (Vijayasardahl, *et al.*, 1990, J. Exp. Med. 171(4):1375-1380); high molecular

weight melanoma antigen (Natali, *et al.*, 1987, Cancer 59:55-63) and prostate specific membrane antigen.

In a specific embodiment, an antigen or fragment or derivative thereof specific to a certain tumor is selected for complexing to HPBF and subsequent administration to a patient having that tumor.

In a preferred embodiment, where it is desired to treat or prevent viral diseases, molecules comprising epitopes of known viruses are used. For example, such antigenic epitopes may be prepared from viruses including, but not limited to, hepatitis type A, hepatitis type B, hepatitis type C, influenza, varicella, adenovirus, herpes simplex type I (HSV-I), herpes simplex type II (HSV-II), rinderpest, rhinovirus, echovirus, rotavirus, respiratory syncytial virus, papilloma virus, papova virus, cytomegalovirus, echinovirus, arbovirus, huntavirus, coxsackie virus, mumps virus, measles virus, rubella virus, polio virus, human immunodeficiency virus type I (HIV-I), and human immunodeficiency virus type II (HIV-II).

In another preferred embodiment, where it is desired to treat or prevent bacterial infections, molecules comprising epitopes of known bacteria are used. For example, such antigenic epitopes may be prepared from bacteria including, but not limited to, mycobacteria rickettsia, mycoplasma, neisseria and legionella.

In another preferred embodiment, where it is desired to treat or prevent protozoal infections, molecules comprising epitopes of known protozoa are used. For example, such antigenic epitopes may be prepared from protozoa including, but not limited to, leishmania, kokzidioa, and trypanosoma.

In yet another preferred embodiment, where it is desired to treat or prevent parasitic infections, molecules comprising epitopes of known parasites are used. For example, such antigenic epitopes may be from parasites including, but not limited to, chlamydia and rickettsia.

To determine immunogenicity or antigenicity of a putative antigen by detecting binding to antibody, various immunoassays known in the art can be used, including but not limited to competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in vivo* immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western blots, immunoprecipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. In one aspect, antibody binding is detected by detecting a label on the primary antibody. In another aspect, the primary antibody is detected by detecting binding of a

secondary antibody or reagent to the primary antibody. In a further aspect, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are envisioned for use. In one embodiment for detecting immunogenicity, T cell-mediated responses can be assayed by standard methods, *e.g.*, *in vitro* cytoxicity assays or *in vivo* delayed-type hypersensitivity assays.

Potentially useful antigens, or derivatives thereof, can be identified by various criteria, such as the antigen's involvement in neutralization of a pathogen's infectivity (wherein it is desired to treat or prevent infection by such a pathogen) (Norrby, 1985, *Summary*, in Vaccines 85, Lerner, *et al.* (eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 388-389), type or group specificity, recognition by patients' antisera or immune cells, and/or the demonstration of protective effects of antisera or immune cells specific for the antigen. In addition, where it is desired to treat or prevent a disease caused by pathogen, the antigen's encoded epitope should preferably display a small or no degree of antigenic variation in time or amongst different isolates of the same pathogen.

5.3.3.2 Peptides From Hsp-Peptide Complexes

Antigenic peptides for complexing *in vitro* to HPBFs of the invention can also be obtained from endogenous complexes of peptides and HSPs. Two methods may be used to elute the peptide from an HSP-peptide complex. One approach involves incubating the HSP-peptide complex in the presence of ATP. The other approach involves incubating the complexes in a low pH buffer.

Briefly, the complex of interest is centrifuged through a Centricon 10 assembly (Millipore) to remove any low molecular weight material loosely associated with the complex. The large molecular weight fraction may be removed and analyzed by SDS-PAGE while the low molecular weight may be analyzed by HPLC as described below. In the ATP incubation protocol, the HSP-peptide complex in the large molecular weight fraction is incubated with 10mM ATP for 30 minutes at room temperature. In the low pH protocol, acetic acid or trifluoroacetic acid (TFA) is added to the HSP-peptide complex to give a final concentration of 10% (vol/vol) and the mixture incubated at room temperature or in a boiling water bath or any temperature in between, for 10 minutes (*See*, Van Bleek, *et al.*, 1990, Nature 348:213-216; and Li, *et al.*, 1993, EMBO Journal 12:3143-3151).

The resulting samples are centrifuged through a Centricon10 assembly as mentioned previously. The high and low molecular weight fractions are recovered. The remaining large molecular weight HSP-peptide complexes can be reincubated with ATP or low pH to remove any remaining peptides.

The resulting lower molecular weight fractions are pooled, concentrated by evaporation and dissolved in 0.1% TFA. The dissolved material is then fractionated by reverse phase high pressure liquid chromatography (HPLC) using for example a VYDAC C18 reverse phase column equilibrated with 0.1% TFA. The bound material is then eluted at a flow rate of about 0.8 ml/min by developing the column with a linear gradient of 0 to 80% acetonitrile in 0.1% TFA. The elution of the peptides can be monitored by OD_{210} and the fractions containing the peptides collected.

5.3.3.3 Peptides from MHC–Peptide Complexes

Peptides bound to MHC molecules *in vivo* can also be used *in vitro* to form complexes with HPBFs of the invention. The isolation of potentially immunogenic peptides from MHC molecules is well known in the art and so is not described in detail herein (*see*, Falk, *et al.*, 1990, Nature 348:248-251; Rotzsche, at al., 1990, Nature 348:252-254; Elliott, *et al.*, 1990, Nature 348:191-197; Falk, *et al.*, 1991, Nature 351:290-296; Demotz, *et al.*, 1989, Nature 343:682-684; Rotzsche, *et al.*, 1990, Science 249:283-287), the disclosures of which are incorporated herein by reference.

Briefly, MHC-peptide complexes may be isolated by a conventional immunoaffinity procedure. The peptides then may be eluted from the MHC-peptide complex by incubating the complexes in the presence of about 0.1% TFA in acetonitrile. The eluted peptides may be fractionated and purified by reverse phase HPLC, as before.

5.3.3.4 Synthetic Peptides

The amino acid sequences of the peptides eluted from MHC molecules or HSPs may be determined either by manual or automated amino acid sequencing techniques well known in the art. Once the amino acid sequence of a potentially protective peptide has been determined, the peptide may be synthesized in using conventional peptide synthesis or other protocols well known in the art.

Peptides having the same amino acid sequence as those isolated above may be synthesized by solid-phase peptide synthesis using procedures similar to those described by Merrifield, 1963, J. Am. Chem. Soc., 85:2149. During synthesis, N-α-protected amino acids having protected side chains are added stepwise to a growing polypeptide chain linked by its C-terminal and to an insoluble polymeric support i.e., polystyrene beads. The peptides are synthesized by linking an amino group of an N-α-deprotected amino acid to an α-carboxyl group of an N-α-protected amino acid that has been activated by reacting it with a reagent such as dicyclohexylcarbodiimide. The attachment of a free amino group to the activated carboxyl leads to peptide bond formation. The most commonly used N-α-protecting groups include Boc which is acid labile and Fmoc which is base labile. Details of

appropriate chemistries, resins, protecting groups, protected amino acids and reagents are well known in the art and so are not discussed in detail herein (*See*, Atherton, *et al.*, 1989, Solid Phase Peptide Synthesis: A Practical Approach, IRL Press, and Bodanszky, 1993, Peptide Chemistry, A Practical Textbook, 2nd Ed., Springer-Verlag).

Purification of the resulting peptides is accomplished using conventional procedures, such as preparative HPLC using gel permeation, partition and/or ion exchange chromatography. The choice of appropriate matrices and buffers are well known in the art and so are not described in detail herein.

5.3.3.5 Recombinantly Produced Antigens

In a particular embodiment of the invention, a nucleotide sequence encoding a protein antigen or portions thereof can be introduced into a host cell for production of the antigen. The nucleotide sequence encoding any antigenic protein can be obtained and cloned into an expression vector for expression essentially by the same methods described for the cloning and expression of a nucleotide sequence encoding an HSP peptide-binding fragment. The techniques are described in Sections 5.2.1.1 and 5.2.1.2 and are well known in the art. The recombinant antigenic protein or portions thereof can be purified by any methods appropriate for the protein, and then used to form complexes with HPBFs *in vitro* as described in Section 5.3.2. Such an HPBF-antigen complex can be used as a vaccine to stimulate an immune response against the antigenic protein in a subject for the purpose of treatment or prevention of infectious diseases or cancer.

5.4 Therapeutic Applications of Noncovalent Complexes of HSP Peptide-Binding Fragments and Antigenic Molecules

The present invention encompasses the use of HPBFs in methods for treatment of and prevention of infectious diseases and cancer. In various embodiments described in detail herein, an effective amount of a HSP peptide-binding fragment in a noncovalent complex with an antigenic molecule is administered to a patient for therapeutic purposes.

5.4.1 Prevention and Treatment of Infectious Diseases

For treatment and prevention of infectious disease, HPBF complexes are prepared from a cell that displays the antigenicity of an antigen of an infectious agent or pathogenic agent, and used as vaccines against the infectious disease. As will be appreciated by those skilled in the art, the protocols described herein may be used to isolate HPBF–peptide complexes from any cell that displays the antigenicity of an antigen of the infectious agent. For example, cells may be infected by the infectious agent itself, or

alternatively, cells may be infected by or engineered to express an attenuated form of the infectious agent or a non-pathogenic or replication-deficient variant of the pathogen. In one embodiment, HPBF complexes can be prepared from cells infected with non-infectious or non-pathogenic forms of the infectious agent (*e.g.*, by use of a helper infectious agent). In another embodiment, the HPBF complexes of the invention may be prepared from cells infected with an intracellular pathogen. In another embodiment, HPBF-complexes can be prepared from cells that have been transformed by an intracellular pathogen. For example, immunogenic HSP peptide-binding fragments complexes may be isolated from eukaryotic cells transformed with a transforming virus such as SV40.

A preferred method for treatment or prevention of an infectious disease comprises introducing into a cell that displays the antigenicity of an infectious agent an expressible HPBF gene sequence, preferably as an expression gene construct. The HPBF gene sequence is manipulated by methods described above in Section 5.2.1.1, so that the HPBF gene sequence, in the form of an expression construct, located extrachromosomally or integrated in the chromosome, is suitable for expression of the HPBF in the recombinant cells. The recombinant cells containing the expression gene constructs are cultured under conditions such that HPBFs encoded by the expression gene construct are expressed. Complexes of HPBFs noncovalently associated with antigenic peptides of the infectious agent are purified from the cell culture or culture medium by the methods described in Section 5.2. Exemplary methods of therapeutic and prophylactic uses of HSP-peptide complexes against intracellular pathogens have also been described in PCT Publications WO 95/24923, dated September 21, 1995, and WO 97/10001, dated March 20, 1997.

In various embodiments, HPBF complexes are prepared from a cell genetically manipulated to express an HPBF, for example, tissues, isolated cells or immortalized eukaryotic cell lines infected with an intracellular pathogen. When immortalized animal cell lines are used as a source of the HPBF–peptide complex, it is important to use cell lines that can be infected with the pathogen of interest. In addition, it is preferable to use cells that are derived from the same species as the intended recipient of the vaccine. Techniques for introducing an expressible form of the HPBF gene sequences into these cell lines are described above in Section 5.3.1.1.

If a pathogen is expected to cause lysis of the host cells, it is preferred to introduce the expressible HPBF gene sequence into the host cell prior to infecting the cells with the pathogen. For example, in order to prepare an HPBF–peptide complex for administration to humans that may be effective against HIV-1, the virus may be propagated in human cells which include, but are not limited to, human CD4+ T cells, HepG2 cells, and U937 promonocytic cells, which have already been transfected with an expressible HPBF sequence. Similarly, influenza viruses may be propagated in, for example, transfected

human fibroblast cell lines and MDCK cells, and mycobacteria may be cultured in, for example, transfected human Schwaan cells. The cell supernatant containing HPBF–peptide complex may be collected just prior to lysis of the host cell.

In a preferred aspect of the invention, the purified HSP peptide-binding fragment complex vaccines may have particular utility in the treatment of human diseases caused by intracellular pathogens. It is appreciated, however, that the vaccines developed using the principles described herein will be useful in treating diseases of other mammals, for example, farm animals including: cattle; horses; sheep; goats; and pigs, and household pets including: cats; and dogs, that similarly are caused by intracellular pathogens.

In accordance with the methods described herein, vaccines may be prepared that stimulate an immune response, in particular a cytotoxic T cell responses, against cells infected with viruses including, but not limited to, hepatitis type A, hepatitis type B, hepatitis type C, influenza, varicella, adenovirus, HSV-I, HSV-II, rinderpest rhinovirus, echovirus, rotavirus, respiratory syncytial virus, papilloma virus, papova virus, cytomegalovirus, echinovirus, arbovirus, huntavirus, coxsachie virus, mumps virus, measles virus, rubella virus, polio virus, HIV-I, and HIV-II. Similarly, vaccines may also be prepared that stimulate cytotoxic T cell responses against cells infected with intracellular bacteria, including, but not limited to, *Mycobacteria, Rickettsia, Mycoplasma, Neisseria* and *Legionella*. In addition, vaccines may also be prepared that stimulate cytotoxic T cell responses against cells infected with intracellular protozoa, including, but not limited to, *Leishmani, Kokzidioa,* and *Trypanosoma*. Furthermore, vaccines may be prepared that stimulate cytotoxic T cell responses against cells infected with intracellular parasites including, but not limited to, *Chlamydia* and *Rickettsia*.

The effect of immunotherapy with modified HPBF–peptide complexes on progression of infectious diseases can be monitored by any methods known to one skilled in the art.

5.4.2 Prevention and Treatment of Cancer

There are many reasons why immunotherapy as provided by the noncovalent HPBF-peptide complexes or recombinant cells expressing HPBFs prepared by the present invention is desired for use in cancer patients. First, if cancer patients are immunosuppressed, and surgery with anesthesia, and subsequent chemotherapy, may worsen the immunosuppression, then with appropriate immunotherapy in the preoperative period, this immunosuppression may be prevented or reversed. This could lead to fewer infectious complications and to accelerated wound healing. Second, tumor bulk is minimal following surgery and immunotherapy is most likely to be effective in this situation. A third

reason is the possibility that tumor cells are shed into the circulation at surgery and effective immunotherapy applied at this time can eliminate these cells.

In a specific embodiment, the preventive and therapeutic utility of the invention is directed at enhancing the immunocompetence of the cancer patient either before surgery, at or after surgery, and at inducing tumor-specific immunity to cancer cells, with the objective being inhibition of cancer, and with the ultimate clinical objective being total cancer regression and eradication.

According to the invention, preferred methods of treatment or prevention of cancer comprise isolating cancer cells from one or more individual, preferably the individual in need of treatment, and introducing into such cells an expressible HPBF gene sequence, preferably as an expression gene construct. The HPBF gene sequence is manipulated by methods described above in Section 5.2.1.1, such that the HPBF gene sequence, in the form of an expression construct, or intrachromosomally integrated, are suitable for expression of the HPBF in the recombinant cells. The recombinant cells containing the expression gene constructs are cultured under conditions such that HPBFs encoded by the expression gene construct are expressed by the recombinant host cells. Complexes of HPBFs noncovalently associated with peptides of the cancer cell are purified from the cell culture or culture medium by the methods described in Section 5.2. Depending on the route of administration, the HPBF-peptide complexes are formulated accordingly as described in Section 5.6, and administered to the individual autologously (*e.g.*, to treat the primary cancer or metastases thereof), or to other individuals who are in need of treatment for cancer of a similar tissue type, or to individuals at enhanced risk of cancer due to familial history or environmental risk factors. Exemplary methods of therapeutic and prophylactic uses of HSP-peptide complexes have also been described in PCT Publications WO 96/10411, dated April 11, 1996, and WO 97/10001, dated March 20, 1997.

For example, treatment with HPBF-peptide complexes prepared as described above may be started any time after surgery. However, if the patient has received chemotherapy, HSP-antigen complexes are usually administered after an interval of four weeks or more so as to allow the immune system to recover. The therapeutic regimen may include weekly injections of the HPBF-antigen complex, dissolved in saline or other physiologically compatible solution. The route and site of injection is varied each time, for example, the first injection is given subcutaneously on the left arm, the second injection on the right arm, the third injection on the left abdominal region, the fourth injection on the right abdominal region, the fifth injection on the left thigh, the sixth injection on the right thigh, etc. The same site is repeated after a gap of one or more injections. In addition, injections are split and each half of the dose is administered at a different site on the same day. Overall, the first four to six injections are given at weekly intervals. Subsequently, two

injections are given at two-week intervals, followed by a regimen of injections at monthly intervals.

Alternatively, recombinant tumor cells expressing HSP–peptide complexes can be used as a vaccine for injection into a patient to stimulate an immune response against the tumor cells or cells bearing tumor antigens. Autologous recombinant tumor cells stably expressing HPBF-peptide complexes are preferred. To determine the appropriate dose, the amount of HPBF-peptide complex produced by the recombinant cells is quantitated, and the number of recombinant cells used for vaccination is adjusted accordingly to assure a consistent level of expression *in vivo*. A preferred dose is the number of recombinant cells that can produce about 100 ng HPBF per 24 hours. For the safety of the patient, the recombinant tumor cells can be irradiated (12000 rad) immediately prior to injection into a patient. Irradiated cells do not proliferate, and can continue to express HPBF-peptide complexes for about 7-10 days which is sufficient to induce an immune response.

Cancers that can be treated or prevented by using noncovalent HSP–peptide complexes prepared by the methods of the present invention include, but not limited to human sarcomas and carcinomas, *e.g.*, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma; leukemias, *e.g.*, acute lymphocytic leukemia and acute myelocytic leukemia (myeloblastic, promyelocytic, myelomonocytic, monocytic and erythroleukemia); chronic leukemia (chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia); and polycythemia vera, lymphoma (Hodgkin's disease and non-Hodgkin's disease), multiple myeloma, Waldenström's macroglobulinemia, and heavy chain disease.

In a specific embodiment, the cancer is metastatic. In another specific embodiment, the patient having a cancer is immunosuppressed by reason of having undergone anti-cancer therapy (*e.g.*, chemotherapy radiation) prior to administration of the

HSP-peptide molecule complexes of the invention. In another specific embodiment, the cancer is a tumor.

The effect of immunotherapy with HPBF-peptide complexes on progression of neoplastic diseases can be monitored by any methods known to one skilled in the art, including but not limited to measuring: a) delayed hypersensitivity as an assessment of cellular immunity; b) activity of cytolytic T-lymphocytes in vitro; c) levels of tumor specific antigens, *e.g.*, carcinoembryonic (CEA) antigens; d) changes in the morphology of tumors using techniques such as a computed tomographic (CT) scan; e) changes in levels of putative biomarkers of risk for a particular cancer in individuals at high risk, and f) changes in the morphology of tumors using a sonogram. Other techniques that can also be used include scintigraphy and endoscopy.

The preventive effect of immunotherapy using HPBF-peptide complexes may also be estimated by determining levels of a putative biomarker for risk of a specific cancer. For example, in individuals at enhanced risk for prostate cancer, serum prostate-specific antigen (PSA) is measured by the procedure described by Brawer *et al.*, 1992, J. Urol. 147:841-845, and Catalona *et al.*, 1993, JAMA 270:948-958; or in individuals at risk for colorectal cancer, CEA is measured by methods known in the art; and in individuals at enhanced risk for breast cancer, 16-α-hydroxylation of estradiol is measured by the procedure described by Schneider *et al.*, 1982, Proc. Natl. Acad. Sci. USA 79:3047-3051. The references cited above are incorporated by reference herein in their entirety.

5.4.3 Combination With Adoptive Immunotherapy

Adoptive immunotherapy refers to a therapeutic approach for treating infectious diseases or cancer in which immune cells are administered to a host with the aim that the cells mediate specific immunity, either directly or indirectly, to the infected cells or tumor cells and/or antigenic components, and result in treatment of the infectious disease or regression of the tumor, as the case may be (see U.S. Patent Application Serial No. 08/527,546, filed September 13, 1995, which is incorporated by reference herein in its entirety). The use of stress protein-peptide complexes for sensitizing antigen presenting cells *in vitro* for use in adoptive immunotherapy is described in PCT publication WO 97/10002, dated March 20, 1997, which is incorporated by reference herein in its entirety. Methods for sensitizing antigen presenting cells (APC) using HPBFs in noncovalently complexes with antigenic (or immunogenic) molecules, for adoptive immunotherapy are described in detail herein.

According to the invention, therapy by administration of HPBF– peptide complexes, using any desired route of administration, is combined with adoptive

immunotherapy using APC sensitized with HPBF–antigenic molecule complexes. The HPBF–peptide complex-sensitized APC can be administered concurrently with HPBF–peptide complexes, or before or after administration of HPBF– peptide complexes. Furthermore, the mode of administration can be varied, including but not limited to, *e.g.*, subcutaneously, intravenously, intraperitoneally, intramuscularly, intradermally or mucosally.

5.4.3.1 Sensitization of Macrophages and Antigen Presenting Cells with HSP Peptide-Binding Fragment – Peptide Complexes

The antigen-presenting cells, including but not limited to macrophages, dendritic cells and B-cells, are preferably obtained by production *in vitro* from stem and progenitor cells from human peripheral blood or bone marrow as described by Inaba *et al.*, 1992, J. Exp. Med. 176:1693-1702. APC can be obtained by any of various methods known in the art. In a preferred aspect human macrophages are used, obtained from human blood cells.

By way of example, but not limitation, macrophages can be obtained as follows: Mononuclear cells are isolated from peripheral blood of a patient (preferably the patient to be treated), by Ficoll-Hypaque gradient centrifugation and are seeded on tissue culture dishes which are pre-coated with the patient's own serum or with other AB+ human serum. The cells are incubated at 37°C for 1 hr, then non-adherent cells are removed by pipetting. To the adherent cells left in the dish, is added cold (4°C) 1 mM EDTA in phosphate-buffered saline and the dishes are left at room temperature for 15 minutes. The cells are harvested, washed with RPMI buffer and suspended in RPMI buffer. Increased numbers of macrophages may be obtained by incubating at 37°C with macrophage-colony stimulating factor (M-CSF); increased numbers of dendritic cells may be obtained by incubating with granulocyte-macrophage-colony stimulating factor (GM-CSF) as described in detail by Inaba, K., *et al.*, 1992, J. Exp. Med. 176:1693-1702.

APC are sensitized with HPBFs noncovalently bound to antigenic molecules by incubating the cells *in vitro* with the complexes. The APC are sensitized with complexes of HPBF and antigenic molecules preferably by incubating *in vitro* with the HPBF–complex at 37°C for 15 minutes to 24 hours. By way of example but not limitation, $4x10^7$ macrophages can be incubated with 10 microgram gp96-peptide complexes per ml or 100 microgram hsp90-peptide complexes per ml at 37°C for 15 minutes-24 hours in 1 ml plain RPMI medium. The cells are washed three times and resuspended in a physiological medium preferably sterile, at a convenient concentration (*e.g.*, $1x10^7$/ml) for injection in a patient. In a preferred embodiment, the antigen presenting cells are autologous to the

patient, that is, the patient into which the sensitized APCs are injected is the patient from which the APC were originally isolated,.

Optionally, the ability of sensitized APC to stimulate, for example, the antigen-specific, class I-restricted cytotoxic T-lymphocytes (CTL) can be monitored by their ability to stimulate CTLs to release tumor necrosis factor, and by their ability to act as targets of such CTLs.

5.4.3.2 Reinfusion of Sensitized APC

The HPBF–antigenic molecule-sensitized APC are reinfused into the patient systemically, preferably intravenously, by conventional clinical procedures. These activated cells are reinfused, preferentially by systemic administration into the autologous patient. Patients generally receive from about 10^6 to about 10^{12} sensitized macrophages, depending on the condition of the patient. In some regimens, patients may optionally receive in addition a suitable dosage of a biological response modifier including but not limited to the cytokines IFN-α, IFN-γ, IL-2, IL-4, IL-6, TNF or other cytokine growth factor.

5.4.4 Determination of Immunogenicity of HSP-Peptide Complexes

In an optional procedure, the purified HPBF–peptide complexes can be assayed for immunogenicity using the mixed lymphocyte target culture assay (MLTC) well known in the art.

By way of example but not limitation, the following procedure can be used. Briefly, mice are injected subcutaneously with the candidate HPBF-peptide complexes. As a positive control another set of mice are immunized with whole cancer cells of the type from which the HPBFs are derived. As a negative control, mice are injected with either HSP-peptide complexes isolated from normal, non-recombinant cells or whole cells (*i.e.*, antigenically distinct from the type of cell from which the HPBFs are derived). The mice are injected twice, 7-10 days apart. The mice are injected twice, 7-10 days apart. Ten days after the last immunization, the spleens are removed and the lymphocytes released. The released lymphocytes may be restimulated subsequently in vitro by the addition of dead cells that expressed the complex of interest.

For example, $8x10^6$ immune spleen cells may be stimulated with $4x10^4$ mitomycin C treated or γ-irradiated (5-10,000 rads) pathogen-infected cells (or cells transfected with a gene encoding an antigen of the infectious agent, as the case may be), or tumor cells in 3ml RPMI medium containing 10% fetal calf serum. In certain cases 33% secondary mixed lymphocyte culture supernatant or interleukin 2 (IL-2) may be included in the culture medium as a source of T cell growth factors (See, Glasebrook *et al.*, 1980, J.

Exp. Med. 151:876). To test the primary cytotoxic T cell response after immunization, spleen cells may be cultured without stimulation. In some experiments spleen cells of the immunized mice may also be restimulated with antigenically distinct cells, to determine the specificity of the cytotoxic T cell response.

Six days later the cultures are tested for cytotoxicity in a 4 hour ^{51}Cr-release assay (See, Palladino *et al.*, 1987, Cancer Res. 47:5074-5079 and Blachere, at al., 1993, J. Immunotherapy 14:352-356). In this assay, the mixed lymphocyte culture is added to a target cell suspension to give different effector:target (E:T) ratios (usually 1:1 to 40:1). The target cells are prelabelled by incubating $1x10^6$ target cells in culture medium containing 200 mCi ^{51}Cr/ml for one hour at 37°C. The cells are washed three times following labeling. Each assay point (E:T ratio) is performed in triplicate and the appropriate controls incorporated to measure spontaneous ^{51}Cr release (no lymphocytes added to assay) and 100% release (cells lysed with detergent). After incubating the cell mixtures for 4 hours, the cells are pelleted by centrifugation at 200g for 5 minutes. The amount of ^{51}Cr released into the supernatant is measured by a gamma counter. The percent cytotoxicity is measured as cpm in the test sample minus spontaneously released cpm divided by the total detergent released cpm minus spontaneously released cpm.

In order to block the MHC class I cascade a concentrated hybridoma supernatant derived from K-44 hybridoma cells (an anti-MHC class I hybridoma) is added to the test samples to a final concentration of 12.5% (Heike *et al.*, 1994, J. Immunotherapy 15:165-174).

An alternative to the chromium-release assay is the ELISPOT assay which measures cytokine release by cytotoxic T cells in vitro after stimulation with specific antigen. Cytokine release is detected by antibodies which are specific for a particular cytokine, such as interleukin-2, tumor necrosis factor α or interferon-γ (for example, see Scheibenbogen *et al.*, 1997, Int. J. Cancer, 71:932-936). The assay is carried out in a microtitre plate which has been pre-coated with an antibody specific for a cytokine of interest which captures the cytokine secreted by T cells. After incubation of T cells for 24-48 hours in the coated wells, the cytotoxic T cells are removed and replaced with a second labelled antibody that recognizes a different epitope on the cytokine. After extensive washing to remove unbound antibody, an enzyme substrate which produces a colored reaction product is added to the plate. The number of cytokine-producing cells is counted under a microscope. This method has the advantages of short assay time, and sensitivity without the need of a large number of cytotoxic T cells.

5.4.5 Monitoring of Effects During Immunotherapy

The effect of immunotherapy with HPBF-antigenic molecule complexes can be monitored by any methods known to one skilled in the art, including but not limited to measuring: a) delayed hypersensitivity as an assessment of cellular immunity; b) activity of cytolytic T-lymphocytes *in vitro*; c) levels of infective agent-agent or tumor-specific antigens, *e.g.*, carcinoembryonic (CEA) antigens. In the case of the use of HPBF complexes for prevention or treatment of cancer, the effect can additionally be monitored by: d) changes in the morphology of tumors using techniques such as a computed tomographic (CT) scan; and e) changes in levels of putative biomarkers of risk for a particular cancer in individuals at high risk, and f) changes in the morphology of tumors using a sonogram.

5.4.5.1 Delayed Hypersensitivity Skin Test

Delayed hypersensitivity skin tests are of great value in the overall immunocompetence and cellular immunity to an antigen. Inability to react to a battery of common skin antigens is termed anergy (Sato *et al.*, 1995, Clin. Immunol. Pathol. 74:35-43).

Proper technique of skin testing requires that the antigens be stored sterile at 4°C, protected from light and reconstituted shorted before use. A 25- or 27-gauge needle ensures intradermal, rather than subcutaneous, administration of antigen. Twenty-four and 48 hours after intradermal administration of the antigen, the largest dimensions of both erythema and induration are measured with a ruler. Hypoactivity to any given antigen or group of antigens is confirmed by testing with higher concentrations of antigen or, in ambiguous circumstances, by a repeat test with an intermediate test.

5.4.5.2 Activity of Cytolytic T Lymphocytes *In Vitro*

The activity of cytolytic T-lymphocytes can be assessed *in vitro* using the following method. Eight x 10^6 peripheral blood-derived T lymphocytes isolated by the Ficoll-Hypaque centrifugation gradient technique, are restimulated with $4x10^4$ mitomycinC-treated tumor cells in 3ml RPMI medium containing 10% fetal calf serum. In some experiments, 33% secondary mixed lymphocyte culture supernatant or IL-2, is included in the culture medium as a source of T cell growth factors.

In order to measure the primary response of cytolytic T-lymphocytes after immunization, T cells are cultured without the stimulator tumor cells. In other experiments, T cells are restimulated with antigenically distinct cells. After six days, the cultures are tested for cytotoxicity in a 4 hour ^{51}Cr-release assay. The spontaneous ^{51}Cr-release of the targets should reach a level less than 20%. For the anti-MHC class I blocking activity, a

tenfold concentrated supernatant of W6/32 hybridoma is added to the test at a final concentration of 12.5% (Heike M., *et al.*, *J. Immunotherapy* 15:165-174).

5.4.5.3 Levels of Tumor Specific Antigens

Although it may not be possible to detect unique tumor antigens on all tumors, many tumors display antigens that distinguish them from normal cells. The monoclonal antibody reagents have permitted the isolation and biochemical characterization of the antigens and have been invaluable diagnostically for distinction of transformed from nontransformed cells and for definition of the cell lineage of transformed cells. The best-characterized human tumor-associated antigens are the oncofetal antigens. These antigens are expressed during embryogenesis, but are absent or very difficult to detect in normal adult tissue. The prototype antigen is carcinoembryonic antigen (CEA), a glycoprotein found on fetal gut and human colon cancer cells, but not on normal adult colon cells. Since CEA is shed from colon carcinoma cells and found in the serum, it was originally thought that the presence of this antigen in the serum could be used to screen patients for colon cancer. However, patients with other tumors, such as pancreatic and breast cancer, also have elevated serum levels of CEA. Therefore, monitoring the fall and rise of CEA levels in cancer patients undergoing therapy has proven useful for predicting tumor progression and responses to treatment.

Several other oncofetal antigens have been useful for diagnosing and monitoring human tumors, *e.g.*, alpha-fetoprotein, an alpha-globulin normally secreted by fetal liver and yolk sac cells, is found in the serum of patients with liver and germinal cell tumors and can be used as a marker of disease status.

5.4.5.4 Computed Tomographic (CT) Scan

CT remains the choice of techniques for the accurate staging of cancers. CT has proved more sensitive and specific than any other imaging techniques for the detection of metastases.

5.4.5.5 Measurement of Putative Biomarkers

The levels of a putative biomarker for risk of a specific cancer are measured to monitor the effect of hsp noncovalently bound to peptide complexes. For example, in individuals at enhanced risk for prostate cancer, serum prostate-specific antigen (PSA) is measured by the procedure described by Brawer, M.K., et. al., 1992, J. Urol. 147:841-845, and Catalona, W.J., *et al.*, 1993, JAMA 270:948-958; and in individuals at enhanced risk for breast cancer, 16-α-hydroxylation of estradiol is measured by the procedure described by Schneider, J. *et al.*, 1982, Proc. Natl. Acad. Sci. ISA 79:3047-3051.

5.4.5.6 Sonogram

A sonogram remains an alternative choice of technique for the accurate staging of cancers.

5.5 Dosage Regimens

It was established in experimental tumor models (Blachere *et al.*, 1993, *J. Immunotherapy* 14:352-356) that the lowest dose of HSP noncovalently bound to peptide complexes which produced tumor regression in mice was between 10 and 25 microgram/mouse weighing 20-25g which is equal to 25mg/25g = 1mg/kg. These amounts should be decreased proportionally with the decreased molecular weight of the HSP binding domain fragments.

Prior art methods extrapolate to human dosages based on body weight and surface area. For example, prior art methods of extrapolating human dosage based on body weight can be carried out as follows: since the conversion factor for converting the mouse dosage to human dosage is Dose Human per kg = Dose Mouse per kg x 12 (*See* Freireich, E.J., *et al.*, 1966, Cancer Chemotherap. Rep. 50:219-244), the effective dose of HSP-peptide complexes in humans weighing 70kg should be 1mg/kg ÷ 12 x 70, *i.e.*, about 6mg (5.8mg).

Drug doses are also given in milligrams per square meter of body surface area because this method rather than body weight achieves a good correlation to certain metabolic and excretionary functions (Shirkey, 1965, JAMA 193:443). Moreover, body surface area can be used as a common denominator for drug dosage in adults and children as well as in different animal species as indicated below in Table 1 (Freireich *et al.*, 1966, Cancer Chemotherap. Rep. 50:219-244).

TABLE 1

REPRESENTATIVE SURFACE AREA TO WEIGHT RATIOS (km) FOR VARIOUS SPECIES[1]

Species	Body Weight (kg)	Surface Area (Sqm)	km Factor
Mouse	0.02	0.0066	3.0
Rat	0.15	0.025	5.9
Monkey	3.0	0.24	12
Dog	8.0	0.40	20
Human, Child	20	0.80	25
Adult	60	1.6	37

Example: To express a mg/kg dose in any given species as the equivalent mg/sq m dose, multiply the dose by the appropriate km factor. In adult human, 100mg/kg is equivalent to 100 mg/kg x 37 kg/sq m = 3700 mg/sq m.

In contrast to both of the above-described prior art methods of determining dosage levels, the present invention provides dosages of the purified complexes of fragments HSPs and antigenic molecules that are much smaller than the dosages estimated by the prior art. For example, according to the invention, an amount of Hsp70 fragment–antigenic molecule complexes and/or gp96 fragment–antigenic molecule complexes is administered that is in the range of about 2 microgram to about 150 micrograms for a human patient, the preferred human dosage being the same as used in a 25g mouse. The dosage for Hsp-90 peptide complexes in a human patient provided by the present invention is in the range of about 10 to 1,000 micrograms, the preferred dosage being 20 micrograms.

The doses recited above are preferably given once weekly for a period of about 4-6 weeks, and the mode or site of administration is preferably varied with each administration. In a preferred example, subcutaneous administrations are given, with each site of administration varied sequentially. Thus, by way of example and not limitation, the first injection may be given subcutaneously on the left arm, the second on the right arm, the third on the left belly, the fourth on the right belly, the fifth on the left thigh, the sixth on the right thigh, etc. The same site may be repeated after a gap of one or more injections. Also,

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[1] Freireich, *et al.*, 1966, Cancer Chemotherap. Rep. 50: 219-244.

split injections may be given. Thus, for example, half the dose may be given in one site and the other half on an other site on the same day.

Alternatively, the mode of administration is sequentially varied, *e.g.*, weekly injections are given in sequence subcutaneously, intramuscularly, intravenously or intraperitoneally.

After 4-6 weeks, further injections are preferably given at two-week intervals over a period of time of one month. Later injections may be given monthly. The pace of later injections may be modified, depending upon the patient's clinical progress and responsiveness to the immunotherapy.

The invention is illustrated by non-limiting examples in Sections 6 and 7.

5.6 Formulation

Noncovalent complexes of HPBFs and antigenic proteins or peptides purified by the methods of the invention may be formulated into pharmaceutical preparations for administration to mammals for treatment or prevention of infectious diseases or cancer. Drug solubility and the site of absorption are factors which should be considered when choosing the route of administration of a therapeutic agent.

HPBF-antigenic molecule complexes of the invention may optionally be administered with one or more adjuvants in order to enhance the immunological response. For example, depending on the host species, adjuvants which may be used include, but are not limited to: mineral salts or mineral gels such as aluminum hydroxide, aluminum phosphate, and calcium phosphate; surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, and dinitrophenol; immunostimulatory molecules, such as cytokines, saponins, muramyl dipeptides and tripeptide derivatives, CpG dinucleotides, CpG oligonucleotides, monophosphoryl Lipid A, and polyphosphazenes; particulate and microparticulate adjuvant, such as emulsions, liposomes, virosomes, cochleates; or an immune stimulating complex mucosal adjuvants, Freund's (complete and incomplete, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.).

HPBF-antigenic molecule complexes of the invention may be administered using any desired route of administration, including but not limited to, *e.g.*, subcutaneously, intravenously or intramuscularly, although intradermally or mucosally is preferred. Advantages of intradermal or mucosal administration include use of lower doses and rapid absorption, respectively. Mucosal routes of administration include, but are not limited to, oral, rectal and nasal administration. Preparations for mucosal administrations are suitable in various formulations as described below. The route of administration can be varied during a course of treatment. Preferred dosages, routes of administration and therapeutic

regimens for complexes of peptides and naturally occurring HSPs are described in PCT International patent applications published as WO 96/10411 and WO 97/10001, which are incorporated by reference herein in their entireties.

Compositions comprising noncovalent complexes formulated in a compatible pharmaceutical carrier may be prepared, packaged, and labeled for treatment of the indicated infectious disease or tumor. In preferred aspects, an amount of HPBF complex is administered to a human that is in the range of about 2 to 150 μg, preferably 20 to 20 μg, most preferably about 5 μg, given once weekly for about 4-6 weeks, intradermally with the site of administration varied sequentially.

If the complex is water-soluble, then it may be formulated in an appropriate buffer, for example, phosphate buffered saline or other physiologically compatible solutions. Alternatively, if the resulting complex has poor solubility in aqueous solvents, then it may be formulated with a non-ionic surfactant such as Tween, or polyethylene glycol. Thus, the noncovalent complexes and their physiologically acceptable solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral, rectal administration or, in the case of tumors, directly injected into a solid tumor.

For oral administration, the pharmaceutical preparation may be in liquid form, for example, solutions, syrups or suspensions, or may be presented as a drug product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, or fractionated vegetable oils); and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.*, pregelatinized maize starch, polyvinyl pyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (*e.g.*, magnesium stearate, talc or silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulphate). The tablets may be coated by methods well-known in the art.

Preparations for oral administration may be suitably formulated to give controlled release of the complexes. Such compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the complexes may be conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane,

dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the complexes and a suitable powder base such as lactose or starch.

The complexes may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

The complexes may also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the complexes may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example, subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the complexes may be formulated with suitable polymeric or hydrophobic materials (for example, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophilic drugs.

The complexes may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the noncovalent complexes. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

The invention also provides kits for carrying out the therapeutic regimens of the invention. Such kits comprise in one or more containers therapeutically or prophylactically effective amounts of the noncovalent HPBF-peptide complexes in pharmaceutically acceptable form. The HPBF-peptide complexes in a vial of a kit of the invention may be in the form of a pharmaceutically acceptable solution, e.g., in combination with sterile saline, dextrose solution, or buffered solution, or other pharmaceutically acceptable sterile fluid. Alternatively, the complex may be lyophilized or desiccated; in this instance, the kit optionally further comprises in a container a pharmaceutically acceptable solution (e.g., saline, dextrose solution, etc.), preferably sterile, to reconstitute the complex to form a solution for injection purposes.

In another embodiment, a kit of the invention further comprises a needle or syringe, preferably packaged in sterile form, for injecting the complex, and/or a packaged alcohol pad. Instructions are optionally included for administration of HPBF-peptide complexes by a clinician or by the patient.

6. EXAMPLE: Administration of Hsp Peptide-Binding Fragment-Peptide Complexes in the Treatment of Infectious Disease

Recombinant cells are engineered to express a peptide-binding domain of Hsp70 and either: peptide A, KRQITDLEMNRLGK (SEQ ID NO:9), derived from G protein of vesicular stomatitis virus (VSV); peptide B, LSSLFRPKRRPIYKS (SEQ ID NO:10) (derived from VSV G protein); peptide C, SLSDLRGYVYQGLKSGNVS (SEQ ID NO:11) (derived from VSV nucleoprotein; see Blachere *et al.*, J. Exp. Med. 1997, 186: 1315-1322; Flynn *et al.*, 1989, Science 245:385-90). Mice, or other subject, with VSV are injected with HPBF-peptide complexes derived from such cells, either separately or in combination. The Hsp70 peptide-binding fragment includes amino acid residues a region from the extreme carboxy-terminus of Hsp70, encompassing amino acid residues 391 to 615. The therapeutic regiment of Hsp70 fragment-peptide complexes, includes weekly injections of the Hsp70 fragment-peptide complexes, dissolved in saline or other physiologically compatible solution.

The dosage used for Hsp70 peptide-binding fragment complexes is in the range of 2-150 micrograms, with the preferred dosage being 2-20 micrograms. The dosage used for Hsp90 peptide-binding fragment complexes is in the range of 10 to 1,000 micrograms, with the preferred dosage being about 20 micrograms.

The route and site of injection is varied each time, for example, the first injection is given subcutaneously on the left arm, the second injection on the right arm, the third injection on the left abdominal region, the fourth injection on the right abdominal region, the fifth injection on the left thigh, the sixth injection on the right thigh, *etc.* The same site is repeated after a gap of one or more injections. In addition, injections are split and each half of the dose is administered at a different site on the same day.

Overall, the first four to six injections are given at weekly intervals. Subsequently, two injections are given at two-week intervals; followed by a regimen of injections at monthly intervals. The effect of Hsp70 fragment-peptide complex therapy is monitored by measuring: a) delayed hypersensitivity as an assessment of cellular immunity; b) activity of cytolytic T-lymphocytes *in vitro*; c) levels of viral specific antigens; d) changes in the progression of the VSV disease, using techniques such as a computed tomographic (CT) scan.

Depending on the results obtained, as described above Section 5.5, the therapeutic regimen is developed to maintain and/or boost the immunological responses of the subject, with the ultimate goal of achieving complete eradication of the virus and its symptoms.

7. EXAMPLE: Adoptive Transfer of Sensitized Macrophage, in Combination with Administration of Peptide-Binding HSP Peptide-Binding Fragment-Peptide Complexes

Autologous human macrophages are sensitized with the peptide-binding domain of the autologous human Hsp70, noncovalently bound to an antigenic/immunogenic molecule. The peptide-binding domain includes amino acid residues a region from the extreme carboxy-terminus of Hsp70, encompassing amino acid residues 391 to 615. The sensitized macrophages are administered to the human patient at approximately the same time as, or before, or after the administration of the Hsp70 fragment-antigenic molecule complex.

7.1. Materials and Methods

Macrophages are obtained as follows: mononuclear cells are isolated from peripheral blood of the human patient to be treated, by Ficoll-Hypaque gradient centrifugation and are seeded on tissue culture dishes which are pre-coated with the patient's own serum or with other AB+ human serum. The cells are incubated at 37°C for 1 hour, then non-adherent cells are removed by pipetting. To the adherent cells left in the dish, is added cold (4°C) 1mM EDTA in phosphate-buffered saline and the dishes are left at room temperature for 15 minutes. The cells are harvested, washed with RPMI buffer and suspended in RPMI buffer. Increased numbers of macrophages may be obtained by incubating at 37°C with macrophage-colony stimulating factor (M-CSF); increased numbers of dendritic cells may be obtained by incubating with granulocyte-macrophage-colony stimulating factor (GM-CSF) as described in detail by Inaba *et al.*, 1992, J. Exp. Med. 176:1693-1702.

The macrophages ($4x10^7$) are then incubated at 37°C for 3 hr in 1ml RPMI containing 50μg Hsp70 fragment-peptide complexes derived from the autologous tumor or from autologous liver, using methods as described in Section 5.3.1.2. The macrophages are then washed 3 times and resuspended at a concentrate of $1x10^7$/ml in RPMI medium. Two hundred microliters of this suspension is administered as described in the experimental protocol below.

7.2. Treatment of Hepatocellular Carcinoma

Five groups of human patients with hepatocellular carcinoma are injected with autologous macrophages sensitized with Hsp70 fragment-peptide complexes derived from their own tumors post surgery. Treatment with Hsp70 fragment-peptide complexes is started any time after surgery. However, if the patient has received chemotherapy, sensitized macrophages alone or in combination with HPBF-peptide complexes are administered after an interval of four weeks or more so as to allow the immune system to recover. The immunocompetence of the patient is tested by procedures described in Sections 5.4.5, above.

The preferred therapeutic regimen includes weekly injections of the sensitized macrophages in combination with an Hsp-peptide complex dissolved in saline or other physiologically compatible solution. Sensitized macrophages may be administered at approximately the same time with an hsp-peptide complex or one may be administered prior to administration of the other.

The dosage used for Hsp70 fragments or gp96 fragments is in the range of 0.1 to 9 micrograms, with the preferred dosage being 0.5-2.0 micrograms. The dosage used for Hsp90 fragments is in the range of 1 to 100 micrograms, with the preferred dosage being about 2 micrograms.

The site of injection is varied each time, for example, the first injection is given intradermally on the left arm, the second injection intradermally on the right arm, the third injection intradermally on the left abdominal region, the fourth injection intradermally on the right abdominal region, the fifth injection intradermally on the left thigh, the sixth injection intradermally on the right thigh, etc. The same site can be alternatively repeated after a gap of one or more injections. In addition, injections are split and each half of the dose is administered at a different site on the same day.

Overall, the first four to six injections are given at weekly intervals. Subsequently, two injections are given at two-week intervals; followed by a regimen of injections at monthly intervals. The effect of therapy is monitored by measuring: a) delayed hypersensitivity as an assessment of cellular immunity; b) activity of cytolytic T-lymphocytes *in vitro*; c) levels of tumor specific antigens, *e.g.*, carcinoembryonic (CEA) antigens; d) changes in the morphology of tumors using techniques such as a computed tomographic (CT) scan; and/or e) changes in putative biomarkers of risk for a particular cancer in individuals at high risk.

Depending on the results obtained, as described above in Section 5.4.1, the therapeutic regimen may be modified to maintain and/or boost the immunological responses of the patient, with the ultimate goal of achieving tumor regression and complete eradication of cancer cells.

The invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

All references cited herein, including patent applications, patents, and other publications, are incorporated by reference herein in their entireties for all purposes.

WHAT IS CLAIMED IS:

1. A pharmaceutical composition comprising an amount of a molecular complex effective for treatment or prevention of an infectious disease or cancer, and a pharmaceutically acceptable carrier, said molecular complex comprising a heat shock protein peptide-binding fragment noncovalently associated with an antigenic molecule, said heat shock protein peptide-binding fragment comprising a peptide-binding domain that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400, and wherein said antigenic molecule displays the antigenicity of an antigen of an infectious agent or of a cancer cell.

2. The pharmaceutical composition of Claim 1 wherein the heat shock protein is Hsp70, Hsp90, gp96, calreticulin, or PDI.

3. The pharmaceutical composition of Claim 2 wherein the heat shock protein is Hsp70.

4. The pharmaceutical composition of Claim 2 wherein the heat shock protein is Hsp90.

5. The pharmaceutical composition of Claim 2 wherein the heat shock protein is PDI.

6. The pharmaceutical composition of Claim 2 wherein the heat shock protein is gp96.

7. The pharmaceutical composition of Claim 1 wherein the molecular complex is purified.

8. The pharmaceutical composition of Claim 1 wherein said heat shock protein fragment lacks one or more other domains of the heat shock protein.

9. A recombinant cell infected with a pathogen and transformed with a nucleic acid comprising a nucleotide sequence that (i) is operably linked to a promoter, and (ii) encodes a heat shock protein peptide-binding fragment comprising a peptide-binding

domain that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400, which heat shock protein peptide-binding fragment noncovalently associates with an antigenic molecule when said antigenic molecule is present, to form a complex that in sufficient amount is capable of eliciting an immune response to the antigenic molecule.

10. The recombinant cell of Claim 9 wherein said cell is a human cell.

11. A recombinant cancer cell transformed with a nucleic acid comprising a nucleotide sequence that (i) is operably linked to a promoter, and (ii) encodes a heat shock protein peptide-binding fragment comprising a peptide-binding domain that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400, which heat shock protein peptide-binding fragment noncovalently associates with an antigenic molecule when said antigenic molecule is present, to form a complex that in sufficient amount is capable of eliciting an immune response to the antigenic molecule.

12. The recombinant cancer cell of Claim 9 or 11 wherein the cell is a human cell.

13. A recombinant cell transformed with (i) a first nucleic acid comprising a first nucleotide sequence that is operably linked to a first promoter and that encodes a heat shock protein peptide-binding fragment a peptide-binding domain that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400, and (ii) a second nucleic acid comprising a second nucleotide sequence that is operably linked to a second promoter and encodes an antigenic molecule, such that the heat shock protein peptide-binding fragment and the antigenic molecule are expressed within the cell and noncovalently associate with each other to form a complex that in sufficient amount is capable of eliciting an immune response to the antigenic molecule.

14. A pharmaceutical composition comprising the recombinant cell of Claim 13 wherein said heat shock protein fragment lacks one or more other domains of the heat shock protein.

15. A pharmaceutical composition comprising the recombinant cell of any one of Claims 9, 11, or 13 and a pharmaceutically acceptable carrier.

16. A method for preparing a complex of a heat shock protein peptide-binding fragment noncovalently associated with a peptide, said heat shock protein peptide-binding fragment comprising a peptide-binding domain that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400, comprising:

a) culturing cells, transformed with a nucleic acid comprising a nucleotide sequence encoding the heat shock protein peptide-binding fragment and operably linked to a promoter, under conditions such that the heat shock protein peptide-binding fragment is expressed by the cells and associates with peptides of the cells; and

b) recovering a population of complexes of the heat shock protein peptide-binding fragment noncovalently associated with peptides from the cells.

17. A method for preparing a heat shock protein peptide-binding fragment noncovalently associated with peptides derived from one or more antigens of an infectious agent, said heat shock protein peptide-binding fragment comprising a peptide-binding domain that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400, comprising:

a) culturing infected cells, transformed with a nucleic acid comprising a nucleotide sequence encoding the heat shock protein peptide-binding fragment and operably linked to a promoter, under conditions such that the heat shock protein peptide-binding fragment is expressed by the cells and associates with peptides of the cells; and

b) recovering from the cells a population of complexes of the heat shock protein peptide-binding fragment noncovalently associated with peptides derived from the infectious agent.

18. A method for preparing a complex of a heat shock protein peptide-binding fragment noncovalently associated with a peptide, said heat shock protein peptide-binding fragment comprising a peptide-binding domain that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400, said method comprising digesting a preparation of heat shock proteins noncovalently associated with peptides with a protease under conditions and for a length of time sufficient for the formation of peptide-binding fragments of the heat shock protein noncovalently associated with peptides.

19. A method for preparing a complex of a heat shock protein peptide-binding fragment noncovalently associated with a peptide, said heat shock protein peptide-binding fragment comprising a peptide-binding domain that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400, said method comprising:

a) digesting a preparation of heat shock proteins with a protease under conditions and for a length of time sufficient for the formation of peptide-binding fragments of the heat shock protein; and

b) contacting the peptide-binding fragments with peptides under conditions and for a length of time sufficient for the formation of complexes of heat shock protein peptide-binding fragments noncovalently associated with peptides.

20. The method of any one of Claims 16 - 19, further comprising purifying the complexes.

21. The method of any one of Claims 16 - 19, further comprising purifying the complexes by affinity chromatography.

22. A method for preparing *in vitro* complexes of heat shock protein peptide-binding fragments noncovalently associated with one or more antigenic molecules, said heat shock protein peptide-binding fragment comprising a peptide-binding domain that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400, said method comprising incubating a heat shock protein peptide-binding fragment and one or more antigenic molecules under conditions and for a length of time sufficient for the formation of the complexes.

23. The method of Claim 22 wherein the one or more antigenic molecules is a population of peptides from an infected cell or a cancer cell.

24. The method of Claim 22 wherein the one or more antigenic molecules displays the antigenicity of an antigen of an infectious agent or a cancer cell.

25. A method of eliciting an immune response against an antigen in an individual comprising administering to the individual an immunogenic complex of a heat shock protein peptide-binding fragment noncovalently associated with a first antigenic molecule displaying antigenicity of the antigen, said heat shock protein peptide-binding fragment comprising a peptide-binding domain that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400.

26. The method of Claim 25, wherein said heat shock protein fragment lacks one or more other domains of the heat shock protein.

27. The method of Claim 25, further comprising, before, concurrently, or after administration of the immunogenic complex, administering to the individual a composition comprising antigen presenting cells sensitized *in vitro* with a sensitizing amount of a second immunogenic complex consisting essentially of a heat shock protein, or peptide-binding fragment thereof, noncovalently bound to a second antigenic molecule, in which said second antigenic molecule shares at least one antigenic determinant with the first antigenic molecule.

28. A method of treating or preventing an infectious disease in an individual having an infectious disease, or in whom prevention of an infectious disease is desired, comprising administering to the individual an immunogenic complex of a heat shock protein peptide-binding fragment noncovalently associated with a first antigenic molecule, said heat shock protein peptide-binding fragment comprising a peptide-binding domain that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400, wherein the first antigenic molecule displays the antigenicity of an antigen of an infectious agent of the infectious disease.

29. The method of Claim 28, wherein said heat shock protein fragment lacks one or more other domains of the heat shock protein.

30. The method of Claim 28, further comprising, before, concurrently or after administration of the immunogenic complex, administering to the individual a composition comprising antigen presenting cells sensitized *in vitro* with a sensitizing amount of a second complex of a heat shock protein or peptide-binding fragment thereof noncovalently bound to a second antigenic molecule, said second antigenic molecule sharing at least one antigenic determinant with the first antigenic molecule.

31. A method of treating or preventing an infectious disease in a subject having an infectious disease or in whom prevention of an infectious disease is desired comprising:

a) culturing an infected cell transformed with a nucleic acid comprising a nucleotide sequence encoding a heat shock protein peptide-binding fragment, said infected cell displaying the antigenicity of an antigen of an infectious agent of the infectious disease, said heat shock protein peptide-binding fragment comprising a peptide-binding domain that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400, said nucleotide sequence being operably linked to a promoter, under conditions such that the peptide-binding fragment is expressed by the infected cells and associates with peptides of the cell;

b) recovering complexes of the heat shock protein peptide-binding fragments noncovalently associated with peptides from the infected cell; and

c) administering to the subject an amount of the recovered complexes effective to treat or prevent the infectious disease.

32. The method of Claim 31, wherein said heat shock protein fragment lacks one or more other domains of the heat shock protein.

33. The method of Claim 31, further comprising, prior to step (a), the step of obtaining infected cells from the subject and transforming the infected cells with the nucleic acid.

34. The method of Claim 31, further comprising, prior to step (a), the step of obtaining the infected cell from one or more individuals and transforming the infected cells with the nucleic acid, said one or more individuals being different from the subject and having the same type of infectious disease as the subject.

35. A method of treating or preventing an infectious disease in a subject having an infectious disease or in whom prevention of an infectious disease is desired comprising:

a) culturing a recombinant cell transformed with (i) a first nucleic acid encoding a heat shock protein peptide-binding fragment comprising a peptide-binding domain that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400, and (ii) a second nucleic acid encoding an antigenic molecule displaying the antigenicity of an antigen of an infectious agent of the infectious disease;

b) recovering complexes of the heat shock protein peptide-binding fragments noncovalently associated with the antigenic molecule; and

c) administering to the subject an amount of the recovered complexes effective to treat or prevent the infectious disease.

36. The method of Claim 28, 31, or 35, in which the infectious disease is caused by an infectious agent selected from the group consisting of a virus, a bacterium, a fungus, and a parasite.

37. A method of treating or preventing cancer in an individual having a type of cancer or in whom prevention of a type of cancer is desired comprising administering to the individual an immunogenic complex of a heat shock protein peptide-binding fragment noncovalently associated with a first antigenic molecule, said heat shock protein comprising a peptide-binding domain that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400, wherein either (a) the first antigenic molecule displays antigenicity of said type of cancer or a metastasis thereof; or (b) the complex is obtained by recovering complexes from said type of cancer cells or a metastasis thereof that recombinantly express the heat shock protein peptide-binding fragment.

38. The method of Claim 37, further comprising, before, concurrently or after administration of the immunogenic complex, administering to the individual a composition comprising antigen presenting cells sensitized *in vitro* with a sensitizing amount of a second complex of a heat shock protein or peptide-binding fragment thereof noncovalently bound to a second antigenic molecule, said second antigenic molecule sharing at least one antigenic determinant with the first antigenic molecule.

39. A method of treating or preventing cancer in a subject having a type of cancer or in whom prevention of a type of cancer is desired comprising:

a) culturing a cancer cell transformed with a nucleic acid comprising a nucleotide sequence encoding a heat shock protein peptide-binding fragment comprising a peptide-binding domain that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400, said nucleotide sequence being operably linked to a promoter, under conditions such that the peptide-binding fragment is expressed by the cancer cell and associates with peptides of the cell;

b) recovering complexes of the heat shock protein peptide-binding fragments noncovalently associated with peptides from the cancer cell; and

c) administering to the subject an amount of the recovered complexes effective to treat or prevent cancer.

40. The method of Claim 39, further comprising, prior to step (a), the step of obtaining cancer cells from the subject and transforming the cancer cells with the nucleic acid.

41. The method of Claim 39, further comprising, prior to step (a), the step of obtaining cancer cells from one or more individuals and transforming the cancer cells with the nucleic acid, said one or more individuals being different from the subject and having the same type of cancer as the subject.

42. A method of treating or preventing cancer in a subject having a type of cancer or in whom prevention of a type of cancer is desired comprising:

a) culturing a recombinant cell transformed with (i) a first nucleic acid encoding a heat shock protein peptide-binding fragment comprising a peptide-binding domain that is contiguous on the N-terminal side with x number of amino acids that naturally flank the peptide-binding domain on the N-terminal side, and that is contiguous on the C-terminal side with y number of amino acids that naturally flank the peptide-binding domain on the C-terminal side, wherein x plus y is not more than 400, and (ii) a second nucleic acid encoding an antigenic molecule displaying the antigenicity of an antigen of a cancer cell;

b) recovering complexes of the heat shock protein peptide-binding fragments noncovalently associated with the antigenic molecule; and

c) administering to the subject an amount of the recovered complexes effective to treat or prevent cancer.

43. The method of Claim 25, 28, 31, 35, 37, 39, or 42, wherein the peptide-binding domain consists of the peptide-binding domain of Hsp70, Hsp90, gp96, calreticulin, PDI, or a mixture of two or more of the foregoing.

44. The method of Claim 43, wherein the peptide-binding fragment is a human BiP peptide-binding fragment comprising the amino acid sequence from about position 413 to about position 638 of the amino acid sequence shown in FIG. 1B (SEQ ID NO:2).

45. The method of Claim 43, wherein the heat shock protein peptide-binding fragment is a human BiP peptide-binding fragment comprising the amino acid sequence from about position 424 to about position 464 of the amino acid sequence shown in FIG. 1B (SEQ ID NO:2).

46. The method of Claim 43, wherein the heat shock protein peptide-binding fragment is a human hsp71 peptide-binding fragment comprising the amino acid sequence from about position 391 to about position 615 of the amino acid sequence shown in FIG. 1C (SEQ ID NO:3).

47. The method of Claim 43, wherein the heat shock protein peptide-binding fragment is a human hsp71 peptide-binding fragment comprising the amino acid sequence from about position 400 to about position 440 of the amino acid sequence shown in FIG. 1C (SEQ ID NO:3).

48. The method of Claim 43, wherein the heat shock protein peptide-binding fragment is a human hsc70 peptide-binding fragment comprising the amino acid sequence from about position 391 to about position 615 of the amino acid sequence shown in FIG. 1D (SEQ ID NO:4).

49. The method of Claim 43, wherein the heat shock protein peptide-binding fragment is a human hsc70 peptide-binding fragment comprising the amino acid sequence from about position 406 to about position 443 of the amino acid sequence shown in FIG. 1D (SEQ ID NO:4).

50. The method of Claim 43, wherein the heat shock protein peptide-binding fragment is a human hsp86 peptide-binding fragment comprising the amino acid sequence from about position 5 to about position 232 of the amino acid sequence shown in FIG. 2A (SEQ ID NO:5).

51. The method of Claim 43, wherein the heat shock protein peptide-binding fragment is a human hsp84 peptide-binding fragment comprising the amino acid sequence

from about position 5 to about position 232 of the amino acid sequence shown in FIG. 2B (SEQ ID NO:6).

52. The method of Claim 43, wherein the heat shock protein peptide-binding fragment is a human gp96 peptide-binding fragment comprising the amino acid sequence from about position 5 to about position 232 of the amino acid sequence shown in FIG. 2C (SEQ ID NO:7).

53. The method of Claim 43, wherein the heat shock protein peptide-binding fragment is a human PDI protein peptide-binding fragment comprising the amino acid sequence from about position 5 to about position 232 of the amino acid sequence shown in FIG. 3 (SEQ ID NO:8).

54. The method of Claim 43, wherein the heat shock protein peptide-binding fragment is a human PDI protein peptide-binding fragment comprising the amino acid sequence from about position 213 to about position 351 of the amino acid sequence shown in FIG. 3 (SEQ ID NO:8).

55. The method of Claim 43, wherein the heat shock protein peptide-binding fragment is a human PDI protein peptide-binding fragment comprising the amino acid sequence from about position 204 to about position 491 of the amino acid sequence shown in FIG. 3 (SEQ ID NO:8).

56. The method of Claim 43, wherein the heat shock protein peptide-binding fragment is a gp96 peptide-binding fragment comprising the amino acid sequence from about position 615 to about position 658 of the amino acid sequence shown in FIG. 2C (SEQ ID NO:7).

57. The method of Claim 43, wherein the heat shock protein peptide-binding fragment is a gp96 peptide-binding fragment comprising the amino acid sequence from about position 624 to about position 630 of the amino acid sequence shown in FIG. 2C (SEQ ID NO:7).

A.

```
  1   *   10    *   20    *   30    *   40    *   50
  1 GKIIGIDLGTTNSCVAIMDGTTPRVLENAEGDRTTPSIIAYTQDGETLVG
 51 QPAKRQAVTNPQNTLFAIKRLIGRRFQDEEVQRDVSIMPFKIIAADNGDA
101 WVEVKGQKMAPPQISAEVLKKMKKTAEDYLGEPVTEAVITVPAYFNDAQR
151 QATKDAGRIAGLEVKRIINEPTAAALAYGLDKGTGNRTIAVYDLGGGTFD
201 ISIIEIDEVDGEKTFEVLATNGDTHLGGEDFDSRLINYLVEEFKKDQGID
251 LRNDPLAMQRLKEAAEKAKIELSSAQQTDVNLPYITADATGPKHMNIKVT
301 RAKLESLVEDLVNRSIEPLKVALQDAGLSVSDIDDVILVGGQTRMPMVQK
351 KVAEFFGKEPRKDVNPDEAVAIGAAVQGGVLTGDVKDVLLLDVTPLSLGI
401 ETMGGVMTTLIAKNTTIPTKHSQVFSTAEDNQSAVTIHVLQGERKRAADN
451 KSLGQFNLDGINPAPRGMPQIEVTFDIDADGILHVSAKDKNSGKEQKITI
501 KASSGLNEDEIQKMVRDAEANAEADRKFEELVQTRNQGDHLLHSTRKQVE
551 EAGDKLPADDKTAIESALTALETALKGEDKAAIEAKMQELAQVSQKLMEI
601 AQQQHAQQQTAGADASANNAKDDDVVDAEFEEVKDKK
```

B.

```
    1   *   10    *   20    *   30    *   40    *   50
  1 MKLSLVAAMLLLLSAARAEEEDKKEDVGTVVGIDLGTTYSCVGVFKNGRV
 51 EIIANDQGNRITPSYVAFTPEGERLIGDAAKNQLTSNPENTVFDAKRLIG
101 RTWNDPSVQQDIKFLPFKVVEKKTKPYIQVDIGGGQTKTFAPEEISAMVL
151 TKMKETAEAYLGKKVTHAVVTVPAYFNDAQRQATKDAGTIAGLNVMRIIN
201 EPTAAAIAYGLDKREGEKNILVFDLGGGTFDVSLLTIDNGVFEVVATNGD
251 THLGGEDFDQRVMEHFIKLYKKKTGKDVRKDNRAVQKLRREVEKAKALSS
301 QHQARIEIESFYEGEDFSETLTRAKFEELNMDLFRSTMKPVQKVLEDSDL
351 KKSDIDEIVLVGGSTRIPKIQQLVKEFFNGKEPSRGINPDEAVAYGAAVQ
401 AGVLSGDQDTGDLVLLHVCPLTLGIETVGGVMTKLIPSNTVVPTKNSQIF
451 STASDNQPTVTIKVYEGERPLTKDNHLLGTFDLTGIPPAPRGVPQIEVTF
501 EIDVNGILRVTAEDKGTGNKNKITITNDQNRLTPEEIERMVNDAEKFAEE
551 DKKLKERIDTRNELESYAYSLKNQIGDKEKLGGKLSSEDKETMEKAVEEK
601 IEWLESHQDADIEDFKAKKKELEEIVQPIISKLYGSAGPPPTGEEDTAEK
651 DEL
```

FIG. 1 A/B

C.

```
      1   *   10    *   20    *   30    *   40    *   50
  1 MAKAAAIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERL
 51 IGDAAKNQVALNPQNTVFDAKRLIGRKFGDPVVQSDMKHWPFQVINDGDK
101 PKVQVSYKGETKAFYPEEISSMVLTKMKEIAEAYLGYPVTNAVITVPAYF
151 NDSQRQATKDAGVIAGLNVLRIINEPTAAAIAYGLDRTGKGERNVLIFDL
201 GGGTFDVSILTIDDGIFEVKATAGDTHLGGEDFDNRLVNHFVEEFKRKHK
251 KDISQNKRAVRRLRTACERAKRTLSSSTQASLEIDSLFEGIDFYTSITRA
301 RFEELCSDLFRSTLEPVEKALRDAKLDKAQIHDLVLVGGSTRIPKVQKLL
351 QDFFNGRDLNKSINPDEAVAYGAAVQAAILMGDKSENVQDLLLLDVAPLS
401 LGLETAGGVMTALIKRNSTIPTKQTQIFTTYSDNQPGVLIQVYEGERAMT
451 KDNNLLGRFELSGIPPAPRGVPQIEVTFDIDANGILNVTATDKSTGKANK
501 ITITNDKGRLSKEEIERMVQEAEKYKAEDEVQRERVSAKNALESYAFNMK
551 SAVEDEGLKGKISEADKKKVLDKCQEVISWLDANTLAEKDEFEHKRKELE
601 QVCNPIISGLYQGAGGPGPGGFGAQGPKGGSGSGPTIEEVD
```

D.

```
  1 MSKGPAVGID LGTTYSCVGV FQHGKVEIIA NDQGNRTTPS YVAFTDTERL IGDAAKNQVA
 61 MNPTNTVFDA KRLIGRRFDD AVVQSDMKHW PFMVVNDAGR PKVQVEYKGE TKSFYPEEVS
121 SMVLTKMKEI AEAYLGKTVT NAVVTVPAYF NDSQRQATKD AGTIAGLNVL RIINEPTAAA
181 IAYGLDKKVG AERNVLIFDL GGGTFDVSIL TIEDGIFEVK STAGDTHLGG EDFDNRMVNH
241 FIAEFKRKHK KDISENKRAV RRLRTACERA KRTLSSSTQA SIEIDSLYEG IDFYTSITRA
301 RFEELNADLF RGTLDPVEKA LRDAKLDKSQ IHDIVLVGGS TRIPKIQKLL QDFFNGKELN
361 KSINPDEAVA YGAAVQAAIL SGDKSENVQD LLLLDVTPLS LGIETAGGVM TVLIKRNTTI
421 PTKQTQTFTT YSDNQPGVLI QVYEGERAMT KDNNLLGKFE LTGIPPAPRG VPQIEVTFDI
481 DANGILNVSA VDKSTGKENK ITITNDKGRL SKEDIERMVQ EAEKYKAEDE KQRDKVSSKN
541 SLESYAFNMK ATVEDEKLQG KINDEDKQKI LDKCNEIINW LDKNQTAEKE EFEHQQKELE
601 KVCNPIITKL YQSAGGMPGG MPGGFPGGGA PPSGGASSGP TIEEVD
```

FIG. 1 C/D

A.

```
   1 MPEETQTQDQ PMEEEEVETF AFQAEIAQLM SLIINTFYSN KEIFLRELIS NSSDALDKIR
  61 YESLTDPSKL DSGKELHINL IPNKQDRTLT IVDTGIGMTK ADLINNLGTI AKSGTKAFME
 121 ALQAGADISM IGQFGVGFYS AYLVAEKVTV ITKHNDDEQY AWESSAGGSF TVRTDTGEPM
 181 GRGTKVILHL KEDQTEYLEE RRIKEIVKKH SQFIGYPITL FVEKERDKEV SDDEAEEKED
 241 KEEEKEKEEK ESEDKPEIED VGSDEEEEKK DGDKKKKKKI KEKYIDQEEL NKTKPIWTRN
 301 PDDITNEEYG EFYKSLTNDW EDHLAVKHFS VEGQLEFRAL LFVPRRAPFD LFENRKKKNN
 361 IKLYVRRVFI MDNCEELIPE YLNFIRGVVD SEDLPLNISR EMLQQSKILK VIRKNLVKKC
 421 LELFTELAED KENYKKFYEQ FSKNIKLGIH EDSQNRKKLS ELLRYYTSAS GDEMVSLKDY
 481 CTRMKENQKH IYYITGETKD QVANSAFVER LRKHGLEVIY MIEPIDEYCV QQLKEFEGKT
 541 LVSVTKEGLE LPEDEEEKKK QEEKKTKFEN LCKIMKDILE KKVEKVVVSN RLVTSPCCIV
 601 TSTYGWTANM ERIMKAQALR DNSTMGYMAA KKHLEINPDH SIIETLRQKA EADKNDKSVK
 661 DLVILLYETA LLSSGFSLED PQTHANRIYR MIKLGLGIDE DDPTADDTSA AVTEEMPPLE
 721 GDDDTSRMEE VD
```

B.

```
   1 MPEEVHHGEE EVETFAFQAE IAQLMSLIIN TFYSNKEIFL RELISNASDA LDKIRYESLT
  61 DPSKLDSGKE LKIDIIPNPQ ERTLTLVDTG IGMTKADLIN NLGTIAKSGT KAFMEALQAG
 121 ADISMIGQFG VGFYSAYLVA EKVVVIRKHN DDEQYAWESS AGGSFTVRAD HGEPIGMGTK
 181 VILHLKEDQT EYLEERRVKE VVKKHSQFIG YPITLYLEKE REKEISDDEA EEEKGEKEEE
 241 DKDDEEKPKI EDVGSDEEDD SGKDKKKKTK KIKEKYIDQE ELNKTKPIWT RNPDDITQEE
 301 YGEFYKSLTN DWEDHLAVKH FSVEGQLEFR ALLFIPRRAP FDLFENKKKK NNIKLYVRRV
 361 FIMDSCDELI PEYLNFIRGV VDSEDLPLNI SREMLQQSKI LKVIRKNIVK KCLELFSELA
 421 EDKENYKKFY EAFSKNLKLG IHEDSTNRRR LSELLRYHTS QSGDEMTSLS EYVSRMKETQ
 481 KSIYYITGES KEQVANSAFV ERVRKRGFEV VYMTEPIDEY CVQQLKEFDG KSLVSVTKEG
 541 LELPEDEEEK KKMEESKAKF ENLCKLMKEI LDKKVEKVTI SNRLVSSPCC IVTSTYGWTA
 601 NMERIMKAQA LRDNSTMGYM MAKKHLEINP DHPIVETLRQ KAEADKNDKA VKDLVVLLFE
 661 TALLSSGFSL EDPQTHSNRI YRMIKLGLGI DEDEVAAEEP NAAVPDEIPP LEGDEDASRM
 721 EEVD
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C.

```
   1 MRALWVLGLC CVLLTFGSVR ADDEVDVDGT VEEDLGKSRE GSRTDDEVVQ REEEAIQLDG
  61 LNASQIRELR EKSEKFAFQA EVNRMMKLII NSLYKNKEIF LRELISNASD ALDKIRLISL
 121 TDENALSGNE ELTVKIKCDK EKNLLHVTDT GVGMTREELV KNLGTIAKSG TSEFLNKMTE
 181 AQEDGQSTSE LIGQFGVGFY SAFLVADKVI VTSKHNNDTQ HIWESDSNEF SVIADPRGNT
 241 LGRGTTITLV LKEEASDYLE LDTIKNLVKK YSQFINFPIY VWSSKTETVE EPMEEEEAAK
 301 EEKEESDDEA AVEEEEEEKK PKTKKVEKTV WDWELMNDIK PIWQRPSKEV EEDEYKAFYK
 361 SFSKESDDPM AYIHFTAEGE VTFKSILFVP TSAPRGLFDE YGSKKSDYIK LYVRRVFITD
 421 DFHDMMPKYL NFVKGVVDSD DLPLNVSRET LQQHKLLKVI RKKLVRKTLD MIKKIADDKY
 481 NDTFWKEFGT NIKLGVIEDH SNRTRLAKLL RFQSSHHPTD ITSLDQYVER MKEKQDKIYF
 541 MAGSSRKEAE SSPFVERLLK KGYEVIYLTE PVDEYCIQAL PEFDGKRFQN VAKEGVKFDE
 601 SEKTKESREA VEKEFEPLLN WMKDKALKDK IEKAVVSQRL TESPCALVAS QYGWSGNMER
 661 IMKAQAYQTG KDISTNYYAS QKKTFEINPR HPLIRDMLRR IKEDEDDKTV LDLAVVLFET
 721 ATLRSGYLLP DTKAYGDRIE RMLRLSLNID PDAKVEEEPE EEPEETAEDT TEDTEQDEDE
 781 EMDVGTDEEE ETAKESTAEK DEL
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FIG.2

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  1 MRSFAPWLVS LLGASAVVAA ADTESDVISL DQDTFESFMN EHGLVLAEFF
 51 APWCGHCKAL APKYEEAATE LKAKNIPLVK VDCTAEEDLC RSQGVEGYPT
101 LKIFRGVDSS KPYQGARQTE SIVSYMIKQS LPAVSSVNEE NLEEIKTMDK
151 IVVIGYIPSD DQETYQAFEK YAESQRDNYL FAATDDAAIA KSEGVEQPSI
201 VLYKDFDEKK AVYDGEIEQE AIHSWVKSAS TPLVGEIGPE TYSGYIGAGV
251 PLAYIFAETK EEREKYTEDF KPIAQKHKGA INIATIDAKM FGAHAGNLNL
301 DSQKFPAFAI QDPAKNAKYP YDQAKELNAD EVEKFIQDVL DGKVEPSIKS
351 EPVPESQEGP VTVVVAHSYK DLVIDNDKDV LLEFYAPWCG HCKALAPKYD
401 ELAALYADHP DLAAKVTIAK IDATANDVPD PITGFPTLRL YPAGAKDSPI
451 EYSGSRTVED LANFVKENGK HNVDALNVAS EETQEGGDVT EAAPSATEAE
501 TPAATDDEKA EHDEL
```

FIG.3

SEQUENCE LISTING

<110> Antigenics, Inc.

<120> COMPLEXES OF PEPTIDE BINDING FRAGMENTS OF HEAT-SHOCK PROTEINS AND THEIR USE AS IMMUNOTHERAPEUTIC AGENTS

<130> 8449-135-228

<140> To Be Assigned
<141> 2000-01-12

<160> 11

<170> PatentIn Ver. 2.1

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Ile Met Asp Gly Thr Thr Pro Arg Val Leu Glu Asn Ala Glu Gly Asp
             20                  25                  30

Arg Thr Thr Pro Ser Ile Ile Ala Tyr Thr Gln Asp Gly Glu Thr Leu
         35                  40                  45

Val Gly Gln Pro Ala Lys Arg Gln Ala Val Thr Asn Pro Gln Asn Thr
     50                  55                  60

Leu Phe Ala Ile Lys Arg Leu Ile Gly Arg Arg Phe Gln Asp Glu Glu
 65                  70                  75                  80

Val Gln Arg Asp Val Ser Ile Met Pro Phe Lys Ile Ile Ala Ala Asp
                 85                  90                  95

Asn Gly Asp Ala Trp Val Glu Val Lys Gly Gln Lys Met Ala Pro Pro
            100                 105                 110

Gln Ile Ser Ala Glu Val Leu Lys Lys Met Lys Lys Thr Ala Glu Asp
        115                 120                 125

Tyr Leu Gly Glu Pro Val Thr Glu Ala Val Ile Thr Val Pro Ala Tyr
    130                 135                 140

Phe Asn Asp Ala Gln Arg Gln Ala Thr Lys Asp Ala Gly Arg Ile Ala
145                 150                 155                 160

Gly Leu Glu Val Lys Arg Ile Ile Asn Glu Pro Thr Ala Ala Ala Leu
                165                 170                 175

Ala Tyr Gly Leu Asp Lys Gly Thr Gly Asn Arg Thr Ile Ala Val Tyr
            180                 185                 190
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Asp Leu Gly Gly Gly Thr Phe Asp Ile Ser Ile Ile Glu Ile Asp Glu
        195                 200                 205

Val Asp Gly Glu Lys Thr Phe Glu Val Leu Ala Thr Asn Gly Asp Thr
    210                 215                 220

His Leu Gly Gly Glu Asp Phe Asp Ser Arg Leu Ile Asn Tyr Leu Val
225                 230                 235                 240

Glu Glu Phe Lys Lys Asp Gln Gly Ile Asp Leu Arg Asn Asp Pro Leu
                245                 250                 255

Ala Met Gln Arg Leu Lys Glu Ala Ala Glu Lys Ala Lys Ile Glu Leu
            260                 265                 270

Ser Ser Ala Gln Gln Thr Asp Val Asn Leu Pro Tyr Ile Thr Ala Asp
        275                 280                 285

Ala Thr Gly Pro Lys His Met Asn Ile Lys Val Thr Arg Ala Lys Leu
    290                 295                 300

Glu Ser Leu Val Glu Asp Leu Val Asn Arg Ser Ile Glu Pro Leu Lys
305                 310                 315                 320

Val Ala Leu Gln Asp Ala Gly Leu Ser Val Ser Asp Ile Asp Asp Val
                325                 330                 335

Ile Leu Val Gly Gly Gln Thr Arg Met Pro Met Val Gln Lys Lys Val
            340                 345                 350

Ala Glu Phe Phe Gly Lys Glu Pro Arg Lys Asp Val Asn Pro Asp Glu
        355                 360                 365

Ala Val Ala Ile Gly Ala Ala Val Gln Gly Gly Val Leu Thr Gly Asp
    370                 375                 380

Val Lys Asp Val Leu Leu Leu Asp Val Thr Pro Leu Ser Leu Gly Ile
385                 390                 395                 400

Glu Thr Met Gly Gly Val Met Thr Thr Leu Ile Ala Lys Asn Thr Thr
                405                 410                 415

Ile Pro Thr Lys His Ser Gln Val Phe Ser Thr Ala Glu Asp Asn Gln
            420                 425                 430

Ser Ala Val Thr Ile His Val Leu Gln Gly Glu Arg Lys Arg Ala Ala
        435                 440                 445

Asp Asn Lys Ser Leu Gly Gln Phe Asn Leu Asp Gly Ile Asn Pro Ala
    450                 455                 460

Pro Arg Gly Met Pro Gln Ile Glu Val Thr Phe Asp Ile Asp Ala Asp
465                 470                 475                 480

Gly Ile Leu His Val Ser Ala Lys Asp Lys Asn Ser Gly Lys Glu Gln
                485                 490                 495
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Lys Ile Thr Ile Lys Ala Ser Ser Gly Leu Asn Glu Asp Glu Ile Gln
            500                 505                 510

Lys Met Val Arg Asp Ala Glu Ala Asn Ala Glu Ala Asp Arg Lys Phe
        515                 520                 525

Glu Glu Leu Val Gln Thr Arg Asn Gln Gly Asp His Leu Leu His Ser
    530                 535                 540

Thr Arg Lys Gln Val Glu Glu Ala Gly Asp Lys Leu Pro Ala Asp Asp
545                 550                 555                 560

Lys Thr Ala Ile Glu Ser Ala Leu Thr Ala Leu Glu Thr Ala Leu Lys
                565                 570                 575

Gly Glu Asp Lys Ala Ala Ile Glu Ala Lys Met Gln Glu Leu Ala Gln
            580                 585                 590

Val Ser Gln Lys Leu Met Glu Ile Ala Gln Gln Gln His Ala Gln Gln
        595                 600                 605

Gln Thr Ala Gly Ala Asp Ala Ser Ala Asn Asn Ala Lys Asp Asp Asp
    610                 615                 620

Val Val Asp Ala Glu Phe Glu Glu Val Lys Asp Lys Lys
625                 630                 635
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<211> 653
<212> PRT
<213> Homo sapiens

<400> 2

```
Met Lys Leu Ser Leu Val Ala Ala Met Leu Leu Leu Leu Ser Ala Ala
  1               5                  10                  15

Arg Ala Glu Glu Glu Asp Lys Lys Glu Asp Val Gly Thr Val Val Gly
             20                  25                  30

Ile Asp Leu Gly Thr Thr Tyr Ser Cys Val Gly Val Phe Lys Asn Gly
         35                  40                  45

Arg Val Glu Ile Ile Ala Asn Asp Gln Gly Asn Arg Ile Thr Pro Ser
     50                  55                  60

Tyr Val Ala Phe Thr Pro Glu Gly Glu Arg Leu Ile Gly Asp Ala Ala
 65                  70                  75                  80

Lys Asn Gln Leu Thr Ser Asn Pro Glu Asn Thr Val Phe Asp Ala Lys
                 85                  90                  95

Arg Leu Ile Gly Arg Thr Trp Asn Asp Pro Ser Val Gln Gln Asp Ile
            100                 105                 110

Lys Phe Leu Pro Phe Lys Val Val Glu Lys Lys Thr Lys Pro Tyr Ile
        115                 120                 125
```

```
Gln Val Asp Ile Gly Gly Gly Gln Thr Lys Thr Phe Ala Pro Glu Glu
    130                 135                 140

Ile Ser Ala Met Val Leu Thr Lys Met Lys Glu Thr Ala Glu Ala Tyr
145                 150                 155                 160

Leu Gly Lys Lys Val Thr His Ala Val Val Thr Val Pro Ala Tyr Phe
                165                 170                 175

Asn Asp Ala Gln Arg Gln Ala Thr Lys Asp Ala Gly Thr Ile Ala Gly
            180                 185                 190

Leu Asn Val Met Arg Ile Ile Asn Glu Pro Thr Ala Ala Ala Ile Ala
        195                 200                 205

Tyr Gly Leu Asp Lys Arg Glu Gly Glu Lys Asn Ile Leu Val Phe Asp
    210                 215                 220

Leu Gly Gly Gly Thr Phe Asp Val Ser Leu Leu Thr Ile Asp Asn Gly
225                 230                 235                 240

Val Phe Glu Val Val Ala Thr Asn Gly Asp Thr His Leu Gly Gly Glu
                245                 250                 255

Asp Phe Asp Gln Arg Val Met Glu His Phe Ile Lys Leu Tyr Lys Lys
            260                 265                 270

Lys Thr Gly Lys Asp Val Arg Lys Asp Asn Arg Ala Val Gln Lys Leu
        275                 280                 285

Arg Arg Glu Val Glu Lys Ala Lys Ala Leu Ser Ser Gln His Gln Ala
    290                 295                 300

Arg Ile Glu Ile Glu Ser Phe Tyr Glu Gly Glu Asp Phe Ser Glu Thr
305                 310                 315                 320

Leu Thr Arg Ala Lys Phe Glu Glu Leu Asn Met Asp Leu Phe Arg Ser
                325                 330                 335

Thr Met Lys Pro Val Gln Lys Val Leu Glu Asp Ser Asp Leu Lys Lys
            340                 345                 350

Ser Asp Ile Asp Glu Ile Val Leu Val Gly Gly Ser Thr Arg Ile Pro
        355                 360                 365

Lys Ile Gln Gln Leu Val Lys Glu Phe Phe Asn Gly Lys Glu Pro Ser
    370                 375                 380

Arg Gly Ile Asn Pro Asp Glu Ala Val Ala Tyr Gly Ala Ala Val Gln
385                 390                 395                 400

Ala Gly Val Leu Ser Gly Asp Gln Asp Thr Gly Asp Leu Val Leu Leu
                405                 410                 415

His Val Cys Pro Leu Thr Leu Gly Ile Glu Thr Val Gly Gly Val Met
            420                 425                 430
```

```
Thr Lys Leu Ile Pro Ser Asn Thr Val Val Pro Thr Lys Asn Ser Gln
        435                 440                 445

Ile Phe Ser Thr Ala Ser Asp Asn Gln Pro Thr Val Thr Ile Lys Val
    450                 455                 460

Tyr Glu Gly Glu Arg Pro Leu Thr Lys Asp Asn His Leu Leu Gly Thr
465                 470                 475                 480

Phe Asp Leu Thr Gly Ile Pro Pro Ala Pro Arg Gly Val Pro Gln Ile
                485                 490                 495

Glu Val Thr Phe Glu Ile Asp Val Asn Gly Ile Leu Arg Val Thr Ala
            500                 505                 510

Glu Asp Lys Gly Thr Gly Asn Lys Asn Lys Ile Thr Ile Thr Asn Asp
        515                 520                 525

Gln Asn Arg Leu Thr Pro Glu Glu Ile Glu Arg Met Val Asn Asp Ala
    530                 535                 540

Glu Lys Phe Ala Glu Glu Asp Lys Lys Leu Lys Glu Arg Ile Asp Thr
545                 550                 555                 560

Arg Asn Glu Leu Glu Ser Tyr Ala Tyr Ser Leu Lys Asn Gln Ile Gly
                565                 570                 575

Asp Lys Glu Lys Leu Gly Gly Lys Leu Ser Ser Glu Asp Lys Glu Thr
            580                 585                 590

Met Glu Lys Ala Val Glu Glu Lys Ile Glu Trp Leu Glu Ser His Gln
        595                 600                 605

Asp Ala Asp Ile Glu Asp Phe Lys Ala Lys Lys Lys Glu Leu Glu Glu
    610                 615                 620

Ile Val Gln Pro Ile Ile Ser Lys Leu Tyr Gly Ser Ala Gly Pro Pro
625                 630                 635                 640

Pro Thr Gly Glu Glu Asp Thr Ala Glu Lys Asp Glu Leu
                645                 650
```

<210> 3
<211> 641
<212> PRT
<213> Homo sapiens

<400> 3

```
Met Ala Lys Ala Ala Ala Ile Gly Ile Asp Leu Gly Thr Thr Tyr Ser
  1               5                  10                  15

Cys Val Gly Val Phe Gln His Gly Lys Val Glu Ile Ile Ala Asn Asp
             20                  25                  30

Gln Gly Asn Arg Thr Thr Pro Ser Tyr Val Ala Phe Thr Asp Thr Glu
         35                  40                  45
```

```
Arg Leu Ile Gly Asp Ala Ala Lys Asn Gln Val Ala Leu Asn Pro Gln
     50                  55                  60

Asn Thr Val Phe Asp Ala Lys Arg Leu Ile Gly Arg Lys Phe Gly Asp
 65                  70                  75                  80

Pro Val Val Gln Ser Asp Met Lys His Trp Pro Phe Gln Val Ile Asn
                 85                  90                  95

Asp Gly Asp Lys Pro Lys Val Gln Val Ser Tyr Lys Gly Glu Thr Lys
            100                 105                 110

Ala Phe Tyr Pro Glu Glu Ile Ser Ser Met Val Leu Thr Lys Met Lys
        115                 120                 125

Glu Ile Ala Glu Ala Tyr Leu Gly Tyr Pro Val Thr Asn Ala Val Ile
    130                 135                 140

Thr Val Pro Ala Tyr Phe Asn Asp Ser Gln Arg Gln Ala Thr Lys Asp
145                 150                 155                 160

Ala Gly Val Ile Ala Gly Leu Asn Val Leu Arg Ile Ile Asn Glu Pro
                165                 170                 175

Thr Ala Ala Ala Ile Ala Tyr Gly Leu Asp Arg Thr Gly Lys Gly Glu
            180                 185                 190

Arg Asn Val Leu Ile Phe Asp Leu Gly Gly Gly Thr Phe Asp Val Ser
        195                 200                 205

Ile Leu Thr Ile Asp Asp Gly Ile Phe Glu Val Lys Ala Thr Ala Gly
    210                 215                 220

Asp Thr His Leu Gly Gly Glu Asp Phe Asp Asn Arg Leu Val Asn His
225                 230                 235                 240

Phe Val Glu Glu Phe Lys Arg Lys His Lys Lys Asp Ile Ser Gln Asn
                245                 250                 255

Lys Arg Ala Val Arg Arg Leu Arg Thr Ala Cys Glu Arg Ala Lys Arg
            260                 265                 270

Thr Leu Ser Ser Ser Thr Gln Ala Ser Leu Glu Ile Asp Ser Leu Phe
        275                 280                 285

Glu Gly Ile Asp Phe Tyr Thr Ser Ile Thr Arg Ala Arg Phe Glu Glu
    290                 295                 300

Leu Cys Ser Asp Leu Phe Arg Ser Thr Leu Glu Pro Val Glu Lys Ala
305                 310                 315                 320

Leu Arg Asp Ala Lys Leu Asp Lys Ala Gln Ile His Asp Leu Val Leu
                325                 330                 335

Val Gly Gly Ser Thr Arg Ile Pro Lys Val Gln Lys Leu Leu Gln Asp
            340                 345                 350
```

```
Phe Phe Asn Gly Arg Asp Leu Asn Lys Ser Ile Asn Pro Asp Glu Ala
        355                 360                 365

Val Ala Tyr Gly Ala Ala Val Gln Ala Ala Ile Leu Met Gly Asp Lys
    370                 375                 380

Ser Glu Asn Val Gln Asp Leu Leu Leu Leu Asp Val Ala Pro Leu Ser
385                 390                 395                 400

Leu Gly Leu Glu Thr Ala Gly Gly Val Met Thr Ala Leu Ile Lys Arg
                405                 410                 415

Asn Ser Thr Ile Pro Thr Lys Gln Thr Gln Ile Phe Thr Thr Tyr Ser
            420                 425                 430

Asp Asn Gln Pro Gly Val Leu Ile Gln Val Tyr Glu Gly Glu Arg Ala
        435                 440                 445

Met Thr Lys Asp Asn Asn Leu Leu Gly Arg Phe Glu Leu Ser Gly Ile
    450                 455                 460

Pro Pro Ala Pro Arg Gly Val Pro Gln Ile Glu Val Thr Phe Asp Ile
465                 470                 475                 480

Asp Ala Asn Gly Ile Leu Asn Val Thr Ala Thr Asp Lys Ser Thr Gly
                485                 490                 495

Lys Ala Asn Lys Ile Thr Ile Thr Asn Asp Lys Gly Arg Leu Ser Lys
            500                 505                 510

Glu Glu Ile Glu Arg Met Val Gln Glu Ala Glu Lys Tyr Lys Ala Glu
        515                 520                 525

Asp Glu Val Gln Arg Glu Arg Val Ser Ala Lys Asn Ala Leu Glu Ser
    530                 535                 540

Tyr Ala Phe Asn Met Lys Ser Ala Val Glu Asp Glu Gly Leu Lys Gly
545                 550                 555                 560

Lys Ile Ser Glu Ala Asp Lys Lys Lys Val Leu Asp Lys Cys Gln Glu
                565                 570                 575

Val Ile Ser Trp Leu Asp Ala Asn Thr Leu Ala Glu Lys Asp Glu Phe
            580                 585                 590

Glu His Lys Arg Lys Glu Leu Glu Gln Val Cys Asn Pro Ile Ile Ser
        595                 600                 605

Gly Leu Tyr Gln Gly Ala Gly Gly Pro Gly Pro Gly Gly Phe Gly Ala
    610                 615                 620

Gln Gly Pro Lys Gly Gly Ser Gly Ser Gly Pro Thr Ile Glu Glu Val
625                 630                 635                 640

Asp
```

<210> 4
<211> 646
<212> PRT
<213> Homo sapiens

<400> 4

```
Met Ser Lys Gly Pro Ala Val Gly Ile Asp Leu Gly Thr Thr Tyr Ser
  1               5                  10                  15

Cys Val Gly Val Phe Gln His Gly Lys Val Glu Ile Ile Ala Asn Asp
             20                  25                  30

Gln Gly Asn Arg Thr Thr Pro Ser Tyr Val Ala Phe Thr Asp Thr Glu
         35                  40                  45

Arg Leu Ile Gly Asp Ala Ala Lys Asn Gln Val Ala Met Asn Pro Thr
     50                  55                  60

Asn Thr Val Phe Asp Ala Lys Arg Leu Ile Gly Arg Arg Phe Asp Asp
 65                  70                  75                  80

Ala Val Val Gln Ser Asp Met Lys His Trp Pro Phe Met Val Val Asn
                 85                  90                  95

Asp Ala Gly Arg Pro Lys Val Gln Val Glu Tyr Lys Gly Glu Thr Lys
            100                 105                 110

Ser Phe Tyr Pro Glu Glu Val Ser Ser Met Val Leu Thr Lys Met Lys
        115                 120                 125

Glu Ile Ala Glu Ala Tyr Leu Gly Lys Thr Val Thr Asn Ala Val Val
    130                 135                 140

Thr Val Pro Ala Tyr Phe Asn Asp Ser Gln Arg Gln Ala Thr Lys Asp
145                 150                 155                 160

Ala Gly Thr Ile Ala Gly Leu Asn Val Leu Arg Ile Ile Asn Glu Pro
                165                 170                 175

Thr Ala Ala Ala Ile Ala Tyr Gly Leu Asp Lys Lys Val Gly Ala Glu
            180                 185                 190

Arg Asn Val Leu Ile Phe Asp Leu Gly Gly Gly Thr Phe Asp Val Ser
        195                 200                 205

Ile Leu Thr Ile Glu Asp Gly Ile Phe Glu Val Lys Ser Thr Ala Gly
    210                 215                 220

Asp Thr His Leu Gly Gly Glu Asp Phe Asp Asn Arg Met Val Asn His
225                 230                 235                 240

Phe Ile Ala Glu Phe Lys Arg Lys His Lys Lys Asp Ile Ser Glu Asn
                245                 250                 255

Lys Arg Ala Val Arg Arg Leu Arg Thr Ala Cys Glu Arg Ala Lys Arg
            260                 265                 270
```

```
Thr Leu Ser Ser Ser Thr Gln Ala Ser Ile Glu Ile Asp Ser Leu Tyr
        275                 280                 285

Glu Gly Ile Asp Phe Tyr Thr Ser Ile Thr Arg Ala Arg Phe Glu Glu
    290                 295                 300

Leu Asn Ala Asp Leu Phe Arg Gly Thr Leu Asp Pro Val Glu Lys Ala
305                 310                 315                 320

Leu Arg Asp Ala Lys Leu Asp Lys Ser Gln Ile His Asp Ile Val Leu
                325                 330                 335

Val Gly Gly Ser Thr Arg Ile Pro Lys Ile Gln Lys Leu Leu Gln Asp
            340                 345                 350

Phe Phe Asn Gly Lys Glu Leu Asn Lys Ser Ile Asn Pro Asp Glu Ala
        355                 360                 365

Val Ala Tyr Gly Ala Ala Val Gln Ala Ala Ile Leu Ser Gly Asp Lys
    370                 375                 380

Ser Glu Asn Val Gln Asp Leu Leu Leu Leu Asp Val Thr Pro Leu Ser
385                 390                 395                 400

Leu Gly Ile Glu Thr Ala Gly Gly Val Met Thr Val Leu Ile Lys Arg
                405                 410                 415

Asn Thr Thr Ile Pro Thr Lys Gln Thr Gln Thr Phe Thr Thr Tyr Ser
            420                 425                 430

Asp Asn Gln Pro Gly Val Leu Ile Gln Val Tyr Glu Gly Glu Arg Ala
        435                 440                 445

Met Thr Lys Asp Asn Asn Leu Leu Gly Lys Phe Glu Leu Thr Gly Ile
    450                 455                 460

Pro Pro Ala Pro Arg Gly Val Pro Gln Ile Glu Val Thr Phe Asp Ile
465                 470                 475                 480

Asp Ala Asn Gly Ile Leu Asn Val Ser Ala Val Asp Lys Ser Thr Gly
                485                 490                 495

Lys Glu Asn Lys Ile Thr Ile Thr Asn Asp Lys Gly Arg Leu Ser Lys
            500                 505                 510

Glu Asp Ile Glu Arg Met Val Gln Glu Ala Glu Lys Tyr Lys Ala Glu
        515                 520                 525

Asp Glu Lys Gln Arg Asp Lys Val Ser Ser Lys Asn Ser Leu Glu Ser
    530                 535                 540

Tyr Ala Phe Asn Met Lys Ala Thr Val Glu Asp Glu Lys Leu Gln Gly
545                 550                 555                 560

Lys Ile Asn Asp Glu Asp Lys Gln Lys Ile Leu Asp Lys Cys Asn Glu
                565                 570
        575
```

```
Ile Ile Asn Trp Leu Asp Lys Asn Gln Thr Ala Glu Lys Glu Glu Phe
            580                 585                 590

Glu His Gln Gln Lys Glu Leu Glu Lys Val Cys Asn Pro Ile Ile Thr
        595                 600                 605

Lys Leu Tyr Gln Ser Ala Gly Gly Met Pro Gly Gly Met Pro Gly Gly
    610                 615                 620

Phe Pro Gly Gly Gly Ala Pro Pro Ser Gly Gly Ala Ser Ser Gly Pro
625                 630                 635                 640

Thr Ile Glu Glu Val Asp
                645
```

<210> 5
<211> 732
<212> PRT
<213> Homo sapiens

<400> 5

```
Met Pro Glu Glu Thr Gln Thr Gln Asp Gln Pro Met Glu Glu Glu Glu
  1               5                  10                  15

Val Glu Thr Phe Ala Phe Gln Ala Glu Ile Ala Gln Leu Met Ser Leu
             20                  25                  30

Ile Ile Asn Thr Phe Tyr Ser Asn Lys Glu Ile Phe Leu Arg Glu Leu
         35                  40                  45

Ile Ser Asn Ser Ser Asp Ala Leu Asp Lys Ile Arg Tyr Glu Ser Leu
     50                  55                  60

Thr Asp Pro Ser Lys Leu Asp Ser Gly Lys Glu Leu His Ile Asn Leu
 65                  70                  75                  80

Ile Pro Asn Lys Gln Asp Arg Thr Leu Thr Ile Val Asp Thr Gly Ile
                 85                  90                  95

Gly Met Thr Lys Ala Asp Leu Ile Asn Asn Leu Gly Thr Ile Ala Lys
            100                 105                 110

Ser Gly Thr Lys Ala Phe Met Glu Ala Leu Gln Ala Gly Ala Asp Ile
        115                 120                 125

Ser Met Ile Gly Gln Phe Gly Val Gly Phe Tyr Ser Ala Tyr Leu Val
    130                 135                 140

Ala Glu Lys Val Thr Val Ile Thr Lys His Asn Asp Asp Glu Gln Tyr
145                 150                 155                 160

Ala Trp Glu Ser Ser Ala Gly Gly Ser Phe Thr Val Arg Thr Asp Thr
                165                 170                 175

Gly Glu Pro Met Gly Arg Gly Thr Lys Val Ile Leu His Leu Lys Glu
            180                 185                 190
```

```
Asp Gln Thr Glu Tyr Leu Glu Glu Arg Arg Ile Lys Glu Ile Val Lys
        195                 200                 205

Lys His Ser Gln Phe Ile Gly Tyr Pro Ile Thr Leu Phe Val Glu Lys
    210                 215                 220

Glu Arg Asp Lys Glu Val Ser Asp Asp Glu Ala Glu Glu Lys Glu Asp
225                 230                 235                 240

Lys Glu Glu Glu Lys Glu Lys Glu Glu Lys Glu Ser Glu Asp Lys Pro
                245                 250                 255

Glu Ile Glu Asp Val Gly Ser Asp Glu Glu Glu Glu Lys Lys Asp Gly
            260                 265                 270

Asp Lys Lys Lys Lys Lys Lys Ile Lys Glu Lys Tyr Ile Asp Gln Glu
        275                 280                 285

Glu Leu Asn Lys Thr Lys Pro Ile Trp Thr Arg Asn Pro Asp Asp Ile
    290                 295                 300

Thr Asn Glu Glu Tyr Gly Glu Phe Tyr Lys Ser Leu Thr Asn Asp Trp
305                 310                 315                 320

Glu Asp His Leu Ala Val Lys His Phe Ser Val Glu Gly Gln Leu Glu
                325                 330                 335

Phe Arg Ala Leu Leu Phe Val Pro Arg Arg Ala Pro Phe Asp Leu Phe
            340                 345                 350

Glu Asn Arg Lys Lys Lys Asn Asn Ile Lys Leu Tyr Val Arg Arg Val
        355                 360                 365

Phe Ile Met Asp Asn Cys Glu Glu Leu Ile Pro Glu Tyr Leu Asn Phe
    370                 375                 380

Ile Arg Gly Val Val Asp Ser Glu Asp Leu Pro Leu Asn Ile Ser Arg
385                 390                 395                 400

Glu Met Leu Gln Gln Ser Lys Ile Leu Lys Val Ile Arg Lys Asn Leu
                405                 410                 415

Val Lys Lys Cys Leu Glu Leu Phe Thr Glu Leu Ala Glu Asp Lys Glu
            420                 425                 430

Asn Tyr Lys Lys Phe Tyr Glu Gln Phe Ser Lys Asn Ile Lys Leu Gly
        435                 440                 445

Ile His Glu Asp Ser Gln Asn Arg Lys Lys Leu Ser Glu Leu Leu Arg
    450                 455                 460

Tyr Tyr Thr Ser Ala Ser Gly Asp Glu Met Val Ser Leu Lys Asp Tyr
465                 470                 475                 480

Cys Thr Arg Met Lys Glu Asn Gln Lys His Ile Tyr Tyr Ile Thr Gly
                485                 490
  495
```

```
Glu Thr Lys Asp Gln Val Ala Asn Ser Ala Phe Val Glu Arg Leu Arg
            500                 505                 510

Lys His Gly Leu Glu Val Ile Tyr Met Ile Glu Pro Ile Asp Glu Tyr
        515                 520                 525

Cys Val Gln Gln Leu Lys Glu Phe Glu Gly Lys Thr Leu Val Ser Val
    530                 535                 540

Thr Lys Glu Gly Leu Glu Leu Pro Glu Asp Glu Glu Glu Lys Lys Lys
545                 550                 555                 560

Gln Glu Glu Lys Lys Thr Lys Phe Glu Asn Leu Cys Lys Ile Met Lys
                565                 570                 575

Asp Ile Leu Glu Lys Lys Val Glu Lys Val Val Val Ser Asn Arg Leu
            580                 585                 590

Val Thr Ser Pro Cys Cys Ile Val Thr Ser Thr Tyr Gly Trp Thr Ala
        595                 600                 605

Asn Met Glu Arg Ile Met Lys Ala Gln Ala Leu Arg Asp Asn Ser Thr
    610                 615                 620

Met Gly Tyr Met Ala Ala Lys Lys His Leu Glu Ile Asn Pro Asp His
625                 630                 635                 640

Ser Ile Ile Glu Thr Leu Arg Gln Lys Ala Glu Ala Asp Lys Asn Asp
                645                 650                 655

Lys Ser Val Lys Asp Leu Val Ile Leu Leu Tyr Glu Thr Ala Leu Leu
            660                 665                 670

Ser Ser Gly Phe Ser Leu Glu Asp Pro Gln Thr His Ala Asn Arg Ile
        675                 680                 685

Tyr Arg Met Ile Lys Leu Gly Leu Gly Ile Asp Glu Asp Asp Pro Thr
    690                 695                 700

Ala Asp Asp Thr Ser Ala Ala Val Thr Glu Glu Met Pro Pro Leu Glu
705                 710                 715                 720

Gly Asp Asp Asp Thr Ser Arg Met Glu Glu Val Asp
                725                 730
```

<210> 6
<211> 724
<212> PRT
<213> Homo sapiens

<400> 6

```
Met Pro Glu Glu Val His His Gly Glu Glu Glu Val Glu Thr Phe Ala
  1               5                  10                  15

Phe Gln Ala Glu Ile Ala Gln Leu Met Ser Leu Ile Ile Asn Thr Phe
             20                  25                  30
```

```
Tyr Ser Asn Lys Glu Ile Phe Leu Arg Glu Leu Ile Ser Asn Ala Ser
        35                  40                  45

Asp Ala Leu Asp Lys Ile Arg Tyr Glu Ser Leu Thr Asp Pro Ser Lys
    50                  55                  60

Leu Asp Ser Gly Lys Glu Leu Lys Ile Asp Ile Ile Pro Asn Pro Gln
65                  70                  75                  80

Glu Arg Thr Leu Thr Leu Val Asp Thr Gly Ile Gly Met Thr Lys Ala
                85                  90                  95

Asp Leu Ile Asn Asn Leu Gly Thr Ile Ala Lys Ser Gly Thr Lys Ala
            100                 105                 110

Phe Met Glu Ala Leu Gln Ala Gly Ala Asp Ile Ser Met Ile Gly Gln
        115                 120                 125

Phe Gly Val Gly Phe Tyr Ser Ala Tyr Leu Val Ala Glu Lys Val Val
    130                 135                 140

Val Ile Arg Lys His Asn Asp Asp Glu Gln Tyr Ala Trp Glu Ser Ser
145                 150                 155                 160

Ala Gly Gly Ser Phe Thr Val Arg Ala Asp His Gly Glu Pro Ile Gly
                165                 170                 175

Met Gly Thr Lys Val Ile Leu His Leu Lys Glu Asp Gln Thr Glu Tyr
            180                 185                 190

Leu Glu Glu Arg Arg Val Lys Glu Val Val Lys Lys His Ser Gln Phe
        195                 200                 205

Ile Gly Tyr Pro Ile Thr Leu Tyr Leu Glu Lys Glu Arg Glu Lys Glu
    210                 215                 220

Ile Ser Asp Asp Glu Ala Glu Glu Glu Lys Gly Glu Lys Glu Glu Glu
225                 230                 235                 240

Asp Lys Asp Asp Glu Glu Lys Pro Lys Ile Glu Asp Val Gly Ser Asp
                245                 250                 255

Glu Glu Asp Asp Ser Gly Lys Asp Lys Lys Lys Lys Thr Lys Lys Ile
            260                 265                 270

Lys Glu Lys Tyr Ile Asp Gln Glu Glu Leu Asn Lys Thr Lys Pro Ile
        275                 280                 285

Trp Thr Arg Asn Pro Asp Asp Ile Thr Gln Glu Glu Tyr Gly Glu Phe
    290                 295                 300

Tyr Lys Ser Leu Thr Asn Asp Trp Glu Asp His Leu Ala Val Lys His
305                 310                 315                 320

Phe Ser Val Glu Gly Gln Leu Glu Phe Arg Ala Leu Leu Phe Ile Pro
                325                 330
            335
```

```
Arg Arg Ala Pro Phe Asp Leu Phe Glu Asn Lys Lys Lys Lys Asn Asn
            340                 345                 350

Ile Lys Leu Tyr Val Arg Arg Val Phe Ile Met Asp Ser Cys Asp Glu
        355                 360                 365

Leu Ile Pro Glu Tyr Leu Asn Phe Ile Arg Gly Val Val Asp Ser Glu
    370                 375                 380

Asp Leu Pro Leu Asn Ile Ser Arg Glu Met Leu Gln Gln Ser Lys Ile
385                 390                 395                 400

Leu Lys Val Ile Arg Lys Asn Ile Val Lys Lys Cys Leu Glu Leu Phe
                405                 410                 415

Ser Glu Leu Ala Glu Asp Lys Glu Asn Tyr Lys Lys Phe Tyr Glu Ala
            420                 425                 430

Phe Ser Lys Asn Leu Lys Leu Gly Ile His Glu Asp Ser Thr Asn Arg
        435                 440                 445

Arg Arg Leu Ser Glu Leu Leu Arg Tyr His Thr Ser Gln Ser Gly Asp
    450                 455                 460

Glu Met Thr Ser Leu Ser Glu Tyr Val Ser Arg Met Lys Glu Thr Gln
465                 470                 475                 480

Lys Ser Ile Tyr Tyr Ile Thr Gly Glu Ser Lys Glu Gln Val Ala Asn
                485                 490                 495

Ser Ala Phe Val Glu Arg Val Arg Lys Arg Gly Phe Glu Val Val Tyr
            500                 505                 510

Met Thr Glu Pro Ile Asp Glu Tyr Cys Val Gln Gln Leu Lys Glu Phe
        515                 520                 525

Asp Gly Lys Ser Leu Val Ser Val Thr Lys Glu Gly Leu Glu Leu Pro
    530                 535                 540

Glu Asp Glu Glu Glu Lys Lys Lys Met Glu Glu Ser Lys Ala Lys Phe
545                 550                 555                 560

Glu Asn Leu Cys Lys Leu Met Lys Glu Ile Leu Asp Lys Lys Val Glu
                565                 570                 575

Lys Val Thr Ile Ser Asn Arg Leu Val Ser Ser Pro Cys Cys Ile Val
            580                 585                 590

Thr Ser Thr Tyr Gly Trp Thr Ala Asn Met Glu Arg Ile Met Lys Ala
        595                 600                 605

Gln Ala Leu Arg Asp Asn Ser Thr Met Gly Tyr Met Met Ala Lys Lys
    610                 615                 620

His Leu Glu Ile Asn Pro Asp His Pro Ile Val Glu Thr Leu Arg Gln
625                 630                 635                 640
```

```
Lys Ala Glu Ala Asp Lys Asn Asp Lys Ala Val Lys Asp Leu Val Val
                645                 650                 655

Leu Leu Phe Glu Thr Ala Leu Leu Ser Ser Gly Phe Ser Leu Glu Asp
            660                 665                 670

Pro Gln Thr His Ser Asn Arg Ile Tyr Arg Met Ile Lys Leu Gly Leu
        675                 680                 685

Gly Ile Asp Glu Asp Glu Val Ala Ala Glu Glu Pro Asn Ala Ala Val
    690                 695                 700

Pro Asp Glu Ile Pro Pro Leu Glu Gly Asp Glu Asp Ala Ser Arg Met
705                 710                 715                 720

Glu Glu Val Asp
```

<210> 7
<211> 803
<212> PRT
<213> Homo sapiens

<400> 7

```
Met Arg Ala Leu Trp Val Leu Gly Leu Cys Cys Val Leu Leu Thr Phe
  1               5                  10                  15

Gly Ser Val Arg Ala Asp Asp Glu Val Asp Val Asp Gly Thr Val Glu
             20                  25                  30

Glu Asp Leu Gly Lys Ser Arg Glu Gly Ser Arg Thr Asp Asp Glu Val
         35                  40                  45

Val Gln Arg Glu Glu Glu Ala Ile Gln Leu Asp Gly Leu Asn Ala Ser
     50                  55                  60

Gln Ile Arg Glu Leu Arg Glu Lys Ser Glu Lys Phe Ala Phe Gln Ala
 65                  70                  75                  80

Glu Val Asn Arg Met Met Lys Leu Ile Ile Asn Ser Leu Tyr Lys Asn
                 85                  90                  95

Lys Glu Ile Phe Leu Arg Glu Leu Ile Ser Asn Ala Ser Asp Ala Leu
            100                 105                 110

Asp Lys Ile Arg Leu Ile Ser Leu Thr Asp Glu Asn Ala Leu Ser Gly
        115                 120                 125

Asn Glu Glu Leu Thr Val Lys Ile Lys Cys Asp Lys Glu Lys Asn Leu
    130                 135                 140

Leu His Val Thr Asp Thr Gly Val Gly Met Thr Arg Glu Glu Leu Val
145                 150                 155                 160

Lys Asn Leu Gly Thr Ile Ala Lys Ser Gly Thr Ser Glu Phe Leu Asn
                165                 170                 175
```

```
Lys Met Thr Glu Ala Gln Glu Asp Gly Gln Ser Thr Ser Glu Leu Ile
            180                 185                 190

Gly Gln Phe Gly Val Gly Phe Tyr Ser Ala Phe Leu Val Ala Asp Lys
        195                 200                 205

Val Ile Val Thr Ser Lys His Asn Asn Asp Thr Gln His Ile Trp Glu
    210                 215                 220

Ser Asp Ser Asn Glu Phe Ser Val Ile Ala Asp Pro Arg Gly Asn Thr
225                 230                 235                 240

Leu Gly Arg Gly Thr Thr Ile Thr Leu Val Leu Lys Glu Glu Ala Ser
                245                 250                 255

Asp Tyr Leu Glu Leu Asp Thr Ile Lys Asn Leu Val Lys Lys Tyr Ser
            260                 265                 270

Gln Phe Ile Asn Phe Pro Ile Tyr Val Trp Ser Ser Lys Thr Glu Thr
        275                 280                 285

Val Glu Glu Pro Met Glu Glu Glu Glu Ala Ala Lys Glu Glu Lys Glu
    290                 295                 300

Glu Ser Asp Asp Glu Ala Ala Val Glu Glu Glu Glu Glu Glu Lys Lys
305                 310                 315                 320

Pro Lys Thr Lys Lys Val Glu Lys Thr Val Trp Asp Trp Glu Leu Met
                325                 330                 335

Asn Asp Ile Lys Pro Ile Trp Gln Arg Pro Ser Lys Glu Val Glu Glu
            340                 345                 350

Asp Glu Tyr Lys Ala Phe Tyr Lys Ser Phe Ser Lys Glu Ser Asp Asp
        355                 360                 365

Pro Met Ala Tyr Ile His Phe Thr Ala Glu Gly Glu Val Thr Phe Lys
    370                 375                 380

Ser Ile Leu Phe Val Pro Thr Ser Ala Pro Arg Gly Leu Phe Asp Glu
385                 390                 395                 400

Tyr Gly Ser Lys Lys Ser Asp Tyr Ile Lys Leu Tyr Val Arg Arg Val
                405                 410                 415

Phe Ile Thr Asp Asp Phe His Asp Met Met Pro Lys Tyr Leu Asn Phe
            420                 425                 430

Val Lys Gly Val Val Asp Ser Asp Asp Leu Pro Leu Asn Val Ser Arg
        435                 440                 445

Glu Thr Leu Gln Gln His Lys Leu Leu Lys Val Ile Arg Lys Lys Leu
    450                 455                 460

Val Arg Lys Thr Leu Asp Met Ile Lys Lys Ile Ala Asp Asp Lys Tyr
465                 470                 475                 480
```

```
Asn Asp Thr Phe Trp Lys Glu Phe Gly Thr Asn Ile Lys Leu Gly Val
                485                 490                 495

Ile Glu Asp His Ser Asn Arg Thr Arg Leu Ala Lys Leu Leu Arg Phe
            500                 505                 510

Gln Ser Ser His His Pro Thr Asp Ile Thr Ser Leu Asp Gln Tyr Val
        515                 520                 525

Glu Arg Met Lys Glu Lys Gln Asp Lys Ile Tyr Phe Met Ala Gly Ser
    530                 535                 540

Ser Arg Lys Glu Ala Glu Ser Ser Pro Phe Val Glu Arg Leu Leu Lys
545                 550                 555                 560

Lys Gly Tyr Glu Val Ile Tyr Leu Thr Glu Pro Val Asp Glu Tyr Cys
                565                 570                 575

Ile Gln Ala Leu Pro Glu Phe Asp Gly Lys Arg Phe Gln Asn Val Ala
            580                 585                 590

Lys Glu Gly Val Lys Phe Asp Glu Ser Glu Lys Thr Lys Glu Ser Arg
        595                 600                 605

Glu Ala Val Glu Lys Glu Phe Glu Pro Leu Leu Asn Trp Met Lys Asp
    610                 615                 620

Lys Ala Leu Lys Asp Lys Ile Glu Lys Ala Val Val Ser Gln Arg Leu
625                 630                 635                 640

Thr Glu Ser Pro Cys Ala Leu Val Ala Ser Gln Tyr Gly Trp Ser Gly
                645                 650                 655

Asn Met Glu Arg Ile Met Lys Ala Gln Ala Tyr Gln Thr Gly Lys Asp
            660                 665                 670

Ile Ser Thr Asn Tyr Tyr Ala Ser Gln Lys Lys Thr Phe Glu Ile Asn
        675                 680                 685

Pro Arg His Pro Leu Ile Arg Asp Met Leu Arg Arg Ile Lys Glu Asp
    690                 695                 700

Glu Asp Asp Lys Thr Val Leu Asp Leu Ala Val Val Leu Phe Glu Thr
705                 710                 715                 720

Ala Thr Leu Arg Ser Gly Tyr Leu Leu Pro Asp Thr Lys Ala Tyr Gly
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Asp Arg Ile Glu Arg Met Leu Arg Leu Ser Leu Asn Ile Asp Pro Asp
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Ala Lys Val Glu Glu Glu Pro Glu Glu Glu Pro Glu Glu Thr Ala Glu
        755                 760                 765

Asp Thr Thr Glu Asp Thr Glu Gln Asp Glu Asp Glu Glu Met Asp Val
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Asp Thr Phe Glu Ser Phe Met Asn Glu His Gly Leu Val Leu Ala Glu
         35                  40                  45

Phe Phe Ala Pro Trp Cys Gly His Cys Lys Ala Leu Ala Pro Lys Tyr
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Glu Glu Ala Ala Thr Glu Leu Lys Ala Lys Asn Ile Pro Leu Val Lys
 65                  70                  75                  80

Val Asp Cys Thr Ala Glu Glu Asp Leu Cys Arg Ser Gln Gly Val Glu
                 85                  90                  95

Gly Tyr Pro Thr Leu Lys Ile Phe Arg Gly Val Asp Ser Ser Lys Pro
            100                 105                 110

Tyr Gln Gly Ala Arg Gln Thr Glu Ser Ile Val Ser Tyr Met Ile Lys
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Gln Ser Leu Pro Ala Val Ser Ser Val Asn Glu Glu Asn Leu Glu Glu
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Ile Lys Thr Met Asp Lys Ile Val Val Ile Gly Tyr Ile Pro Ser Asp
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Asp Gln Glu Thr Tyr Gln Ala Phe Glu Lys Tyr Ala Glu Ser Gln Arg
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Trp Val Lys Ser Ala Ser Thr Pro Leu Val Gly Glu Ile Gly Pro Glu
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Ile Ala Gln Lys His Lys Gly Ala Ile Asn Ile Ala Thr Ile Asp Ala
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Lys Met Phe Gly Ala His Ala Gly Asn Leu Asn Leu Asp Ser Gln Lys
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Phe Pro Ala Phe Ala Ile Gln Asp Pro Ala Lys Asn Ala Lys Tyr Pro
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Tyr Asp Gln Ala Lys Glu Leu Asn Ala Asp Glu Val Glu Lys Phe Ile
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Gln Asp Val Leu Asp Gly Lys Val Glu Pro Ser Ile Lys Ser Glu Pro
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Val Pro Glu Ser Gln Glu Gly Pro Val Thr Val Val Val Ala His Ser
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Tyr Lys Asp Leu Val Ile Asp Asn Asp Lys Asp Val Leu Leu Glu Phe
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385                 390                 395                 400

Glu Leu Ala Ala Leu Tyr Ala Asp His Pro Asp Leu Ala Ala Lys Val
                405                 410                 415

Thr Ile Ala Lys Ile Asp Ala Thr Ala Asn Asp Val Pro Asp Pro Ile
            420                 425                 430

Thr Gly Phe Pro Thr Leu Arg Leu Tyr Pro Ala Gly Ala Lys Asp Ser
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Pro Ile Glu Tyr Ser Gly Ser Arg Thr Val Glu Asp Leu Ala Asn Phe
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Val Lys Glu Asn Gly Lys His Asn Val Asp Ala Leu Asn Val Ala Ser
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Asp Glu Leu
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Asn Val Ser

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 July 2001 (26.07.2001)

PCT

(10) International Publication Number
WO 01/52791 A3

(51) International Patent Classification[7]: A61K 39/00, 39/38, C12N 5/06, 5/16, 5/00, 5/02

(21) International Application Number: PCT/US01/01781

(22) International Filing Date: 18 January 2001 (18.01.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/488,393 20 January 2000 (20.01.2000) US

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(81) Designated States *(national)*: AU, CA, JP.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR).

Published:
— *with international search report*

(88) Date of publication of the international search report:
17 January 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/52791 A3

(54) Title: COMPLEXES OF PEPTIDE-BINDING FRAGMENTS OF HEAT SHOCK PROTEINS AND THEIR USE AS IMMUNOTHERAPEUTIC AGENTS

(57) Abstract: The present invention relates to pharmaceutical compositions comprising peptide-binding fragments of heat shock proteins (HSPs) and noncovalent complexes of peptide-binding fragments of HSPs in noncovalent association with antigenic molecules. The invention further relates to methods for the use of such pharmaceutical compositions as immunotherapeutic agents for the treatment and prevention of infectious diseases and cancer.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/01781

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 39/00, 39/38; C12N 5/06, 5/16, 5/00, 5/02
US CL : 424/184.1, 435/330, 435/325

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/184.1, 435/330, 435/325

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
HCAPLUS, WPIDS, MEDLINE, BIOSIS
search terms: heat shock protein, tumor, cancer, immunotherapy, antitumor agent, therapy, complex, vaccine

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	US 6,017,540 A (SRIVASTAVA, et al.) 25 January 2000, see entire document.	1, 2, 11-12, 15-16, 18, 20-21, 25-26, 37-38, 43
Y	WO 97/10001 A1 (FORDHAM UNIVERSITY) 20 March 1997, see entire document.	1, 2, 11-12, 15-16, 18, 20-21, 25, 37, 43
Y	ZHU et al. Structural Analysis of Substrate Binding by the Molecular Chaperone DnaK. Science. 14 June 1996, Vol. 272, pages 1606-1614, see entire document.	1, 2, 11-12, 15-16, 18, 20-21, 25-26, 37-38, 43

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
	09 JUL 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Anthony Caputa Telephone No. 703-308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/01781

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: Please See Continuation Sheet

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/01781

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING Group I, claims 1-8, 11-12, 15, 16, 18-21, 25-27, and 37-57 (in part, as it reads on cancer), drawn to a pharmaceutical composition of a molecular complex for treating or preventing cancer, a recombinant cancer cell transformed with a nucleic acid, and a pharmaceutical composition comprising a recombinant cancer cell, a method for preparing a molecular complex, comprising a heat shock protein peptide-binding fragment noncovalently associated with a peptide, a method of eliciting an immune response using a molecular complex, and a method of treating or preventing cancer using a molecular complex, wherein the antigenic molecule is derived from a cancer agent.

Group II, claims1-10 and 13-15 (in part, as it reads on infectious disease), drawn to a pharmaceutical composition of a molecular complex for treating or preventing an infectious disease, a recombinant cell infected with a pathogen, and a pharmaceutical composition comprising a recombinant cell.

Group III, claims 17, 20, and 21 (claims 20-21in part, as it reads on purifying complexes), drawn to a method for preparing a molecular complex, comprising a heat shock protein peptide-binding fragment noncovalently associated with peptides derived from one or more antigens of infectious agents.

Group IV, claims 22-24, drawn to a method of preparing in vitro complexes of shock protein peptide-binding fragment noncovalently associated with one or more antigenic molecules.

Group V, claims 25-36 and 43-57 (in part, as it reads on infectious disease), drawn to a method of eliciting an immune response using a molecular complex and a method of treating or preventing infectious disease using a molecular complex, wherein the antigenic molecule is derived from an infectious agent.

Continuation of Box II Item 4: 1-8, 11-12, 15, 16, 18-21, 25-27, and 37-57, as it reads on cancer, and Species A, I, and L.

Continuation of B. FIELDS SEARCHED Item 3: HCAPLUS, WPIDS, MEDLINE, BIOSISSearch terms: heat shock protein, tumor, cancer, immunotherapy, antitumor agent, therapy, complex, vaccine